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Studies on the alpha-glucosidase enzyme of Bacillus stearothermophilus, ATCC 7953, a biological indicator test organism

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STUDIES ON THE α-GLUCOSIDASE ENZYME OF *BACILLUS STEAROTHERMOPHILUS*, ATCC 7953, A BIOLOGICAL INDICATOR TEST ORGANISM

Submitted by Heidemarie Albert

for the degree of Doctor of Philosophy

of the University of Bath

1995

Thesis

This research was carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of Dr. C.J. Soper, B. Pharm., MSc., PhD., MRPharmS and Dr. D.J.G. Davies, MSc., PhD., FRPharmS.

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Summary

This thesis details work carried out to characterise the α-glucosidase enzyme associated with spores of *Bacillus stearothermophilus*, ATCC 7953. This enzyme is a normal constituent of spores and vegetative cells of *B.stearothermophilus*, as well as of other bacteria, yeasts and mammalian cells. The survival of the spore-associated enzyme following exposure to "flash" sterilisation conditions appears to correlate well with spore survival. Therefore, the α-glucosidase activity has been used as the basis of Attest ™ Rapid Readout Biological Indicator (3M Healthcare) function.

The introduction describes the Attest ™ Rapid Readout Biological Indicator uses and function. The structure of bacterial spores is detailed, with emphasis on distinguishing features of thermophilic bacilli. The sporulation process is outlined, indicating factors affecting the characteristics of the spores produced. The sequence of events occuring in the activation, germination and outgrowth of spores is described, evaluating mechanisms currently postulated to be involved in these processes. A comparison of spore and vegetative cell enzymes is made, particularly with respect to postulated mechanisms of thermostability.

Chapter 3 describes the initial stages of the work, focusing on characterisation of the enzyme. This involved the extraction of α-glucosidase from spores and vegetative cells, and comparison of various parameters of the two forms of the enzyme. The kinetic parameters, molecular weights and cross-reactivity of the two enzymes were determined, and the effects of temperature and pH on the enzymes were studied.

Chapter 4 details several strategies aimed at optimising the activity of α-glucosidase associated with the spore during their germination and outgrowth in recovery medium. This was approached either by the study of factors affecting the rate and extent of germination of spores in various media, or by investigating the influence of

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INTRODUCTION

1.1 "Flash" sterilisation

"Flash" sterilisation was originally developed for use in operating theatres by Underwood [1], as an emergency procedure for reprocessing of instruments whose sterility has been compromised, and is also used where financial constraints limit the number of expensive articles that are available, and thus necessitate rapid re-sterilisation after use. "Flash" sterilisation (steam sterilisation at 270°F / 132°C, 27 psi) was intended for sterilising unwrapped metal instruments using a 5 minute cycle. Perkins [2] later determined that a 3 minute cycle was adequate for sterilisation of metal instruments. The process has since been extended to include the sterilisation of wrapped items, by increasing the process time to 10 minutes.

Compared to other sterilisation processes, the 3 minute cycle, which is widely used, has a reduced margin of safety. Therefore, especially in this process, a rapid indicator of sterilisation failure is very important. Weekly monitoring of every flash steriliser with a biological indicator and a chemical indicator is the minimum recommendation of the Association for the Advancement of Medical Instrumentation (AAMI), Association of Operating Room Nurses (AORN), Joint Accreditation of Hospitals (JCAH), and the Centres for Disease Control (CDC). A common practice is to monitor the steriliser at the beginning of each day, after a dummy cycle has been run to bring the steriliser up to temperature and clear condensate from the steam line. AAMI, AORN, and CDC also recommend monitoring every load containing implantables or intravascular device [3].

1.2 Biological Indicators

A biological indicator (BI) is a carrier inoculated with a defined number of bacterial spores, contained within a primary pack ready for use, and providing a defined resistance to the specified sterilisation process [4]. Different species and strains of bacterial spores have been used in BIs, having high and consistent resistances to different sterilisation processes. For example, *B.stearothermophilus* spores are used to monitor moist heat sterilisation, *B.subtilis* spores for ethylene oxide, and *B.pumilis* spores are used for radiation monitoring. Requirements of an ideal BI are a high and consistent resistance, linear inactivation kinetics of the spores, ease of preparation, and ease of recovery of any surviving spores. In most cases these requirements have been met. However, in the case of biological monitoring of radiation sterilsation, *Enterococcus faecalis* and *Micrococcus radiodurans* have been found to be much more resistant to the normal sterilising dose of radiation (2.5MRad) than *B.pumilis*.

BIs are used both to validate and monitor the effectiveness of sterilisation processes, but should always be used in combination with physical and chemical monitoring. In moist heat sterilisation, the attainment of specified physical parameters of the process is a more accurate predictor of a satisfactory process than is the use of BIs. For more complex processes, such as ethylene oxide sterilisation, where the process has an increased number of variables, a direct measure of the microbial lethality of the process, *ie.* the use of BIs, is the most satisfactory monitoring method to employ [4].

The use of traditional biological indicators, however, has several limitations, especially relevant to the monitoring of an emergency sterilisation procedure. Their readout time, *ie.* the time taken for any surviving spores to germinate and outgrow in recovery medium, may be up to 7 days, which leads to the use of sterilised products without knowledge of the BI results. When transferring BIs to recovery media, there is a

potential for contamination, and also the possibility of operator error in the interpretation of results [5].

An example of a BI which overcomes these problems is the 1291 Attest ™ Rapid Readout Biological Indicator (3M Healthcare, St. Paul, MN, USA). It is a self-contained BI which has an initial readout time of 1 hour. A self-contained BI is a BI presented in such a way that the primary pack, intended for incubation, contains the growth medium required for recovery. The initial readout time of 1 hour can be supported by a 24 hour confirmation of the result. This biological indicator was developed specifically for use in "flash" sterilisation [6]. However, in practice, BIs used to monitor "flash" sterilisation are still only used as an after-process measurement, since there is no quarantine of products. In spite of this, a rapid result means that potential steriliser malfunction can be dealt with as soon as possible, since a positive fluorescence result can occur prior to any visual colour change and growth after 24 hours incubation, suggesting a marginal sterilisation. This technology has also been applied to monitoring other sterilisation methods, *eg*. ethylene oxide, in a similar way.

1.2.1 1291 Attest ™ Rapid Readout Biological Indicator

Interest in studying the alpha-glucosidase from *B.stearothermophilus* spores comes from the importance of the enzyme in the function of the 1291 Attest ™ Rapid Readout Biological Indicator. It relies on the detection of active alpha-glucosidase following exposure to "flash" sterilisation conditions. Alpha-glucosidase is a normal constituent of *B.stearothermophilus* spores and vegetative cells. It is consistently detectable in viable spores, but it is destroyed at 132°C just after spore kill [6]. Since it remains active just longer than the spore, a positive test result may indicate a marginal sterilisation process, even though spores are not viable.

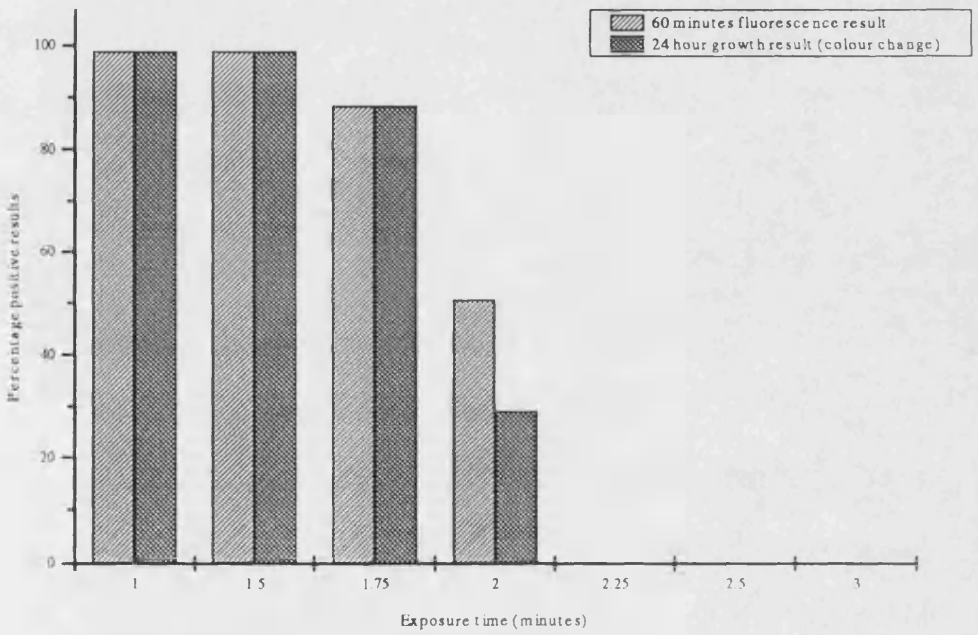
The 1291 Attest ™ Rapid Readout Biological Indicator consists of a dry paper strip containing at least 10^5 spores of *Bacillus stearothermophilus* ATCC 7953 per strip, a modified tryptic soy broth medium, and a dual indicator system, in a self-contained vial (Figure 1.1).

Figure 1.1. 1291 Attest ™ Rapid Readout Biological Indicator Design.

The first indicator consists of a non-fluorescent substrate, 4-methylumbelliferyl-α-D-glucoside (4-MUG), which is converted to a fluorescent product by the action of active alpha-glucosidase enzyme associated with the spores, after incubation for 1 hour at 60°C. The reaction is time-dependent according to the amount of enzyme present. Fluorescence is detected within a few minutes for the positive (unexposed) controls, but at marginal heating times, the full 1 hour incubation is needed for the fluorescent product to be detectable. The indicators are incubated in the Attest

Autoreader (3M) at 60°C. There is no increase in the reliability of the system when results from the fluorescence indicator are read after more than 1 hour incubation.

Figure 1.2. Spore and alpha-glucosidase survival following exposure of Attest Rapid Readout Biological Indicators to gravity steam sterilisation at 132°C.
From 1291 Attest ™ Rapid Readout Biological Indicator Technical Report. Medical-Surgical Products. 3M Healthcare.



The second indicator allows for 24 hour confirmation of the initial result. A pH-sensitive dye, bromocresol purple, turns yellow within 24 hours, due to acid produced by the metabolism of glucose by the spores. This indicates the presence of viable spores, and thus sterilisation failure. No colour change indicates satisfactory sterilisation.

Correlation of the destruction of the alpha-glucosidase activity with spore kill was carried out by incremental exposures of Attest ™ Rapid Readout Biological Indicators to gravity displacement steam sterilisation at 132°C (Figure 1.2). Purified alpha-glucosidase dried onto filter paper strips had very similar characteristics to the spore-associated enzyme in the spore strips.

1.2 Bacterial spore structure

Spores are formed by certain bacteria, mainly members of the genera *Bacillus* and *Clostridium*, when the growth rate of vegetative cells has been reduced as a result of the limitation of an essential nutrient. Spores have certain properties such as resistance to heat, toxic chemicals and desiccation that allow them to survive adverse conditions in which vegetative cells would be destroyed. The spores exist in a dormant state until conditions are such that germination and outgrowth are possible, and vegetative growth can resume. Figure 1.3 shows a diagrammatic representation of the structure of a bacterial spore [7].

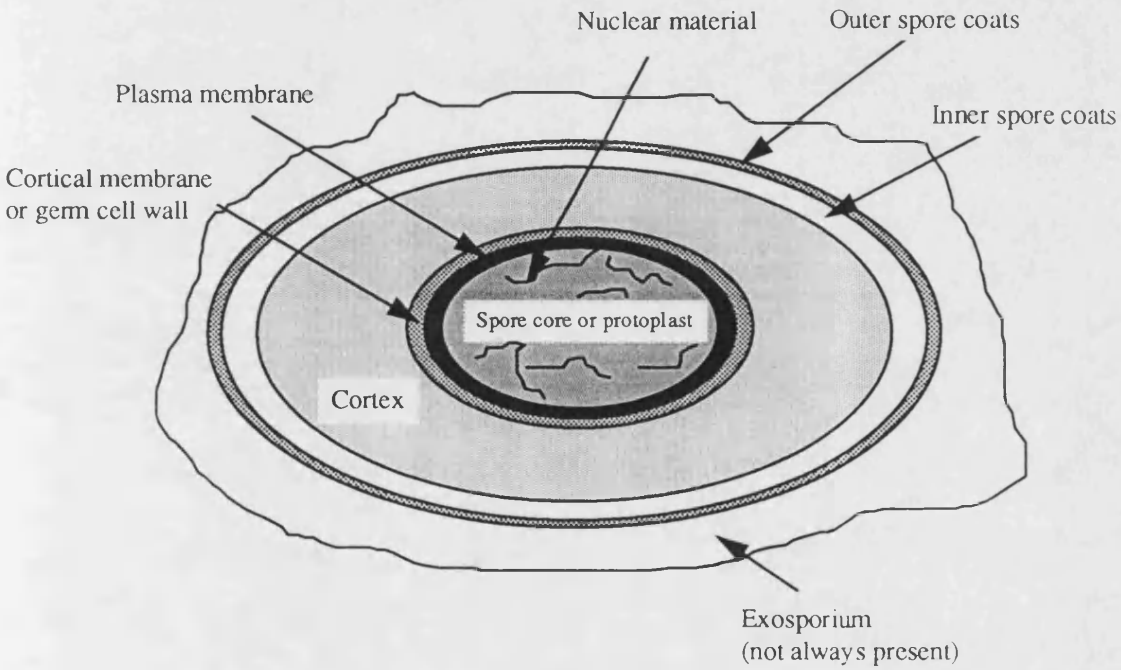
1.2.1 Protoplast (spore core)

This consists of the cytoplasm, nucleoid and the plasma membrane. The core contains DNA, RNA, ribosomes, enzymes, and small amounts of free amino acids. These compounds are in smaller amounts than in the corresponding vegetative cell, but are sufficient for the initial stages of growth, until synthesis of new compounds resumes. The macromolecular composition of the spore core is substantially the same as vegetative cells. However, the composition of small molecules is significantly different [8]. Proteins and nucleic acids account for 50-60% of the dry weight of the core, the remainder being mainly dipicolinic acid and other ions. Fifty percent of the core protein consists of a

group of basic, low molecular weight proteins. They act as a reserve for germination, and contain a high proportion of polar amino acids.

Spores have a very high concentration of calcium and manganese, but relatively low content of potassium and magnesium compared to vegetative cells. Other divalent metal cations accumulated during sporulation include Mn^{2+}, Ni^{2+}, Zn^{2+}, Sr^{2+} and Ba^{2+}.

Figure 1.3. Diagrammatic representation of the structure of a bacterial spore



When these ions are added to the medium at a high concentration, they lower the calcium and DPA content of spores, and the heat resistance is reduced [8]. Dipicolinic acid is localised in the core, mostly in a 1:1 chelate with calcium ions. Although DPA is postulated to have an important role in heat resistance, mutants of *B.cereus* T have been isolated whose spores lack DPA but are fully heat resistant [9].

The spore core has a very low water content, probably less than 20%. The relatively dehydrated core with high concentrations of hydrophilic solutes requires a physical constraint to the osmotic and swelling pressure. This is provided in part by intramolecular bonds between DPA, Ca^{2+} and other core constituents. The remainder of the pressure must be provided by the outer layers of the spore.

Deoxyribonucleotides, which are not needed in the initial stages of germination, and the relatively unstable ribonucleotide triphosphates, are virtually absent from the protoplast. Ribonucleotide di- and mono- phosphates are present in the core, since they are necessary for synthesis of mRNA. Energy requirements of the spore seem to be met by 3-phosphoglyceric acid (3PGA) which is present in the core of bacilli. The enzymes associated with the tricarboxylic acid cycle are absent from the core, and the cytochrome content is very low. However, some energy requirements may be provided by the glycolytic pathway.

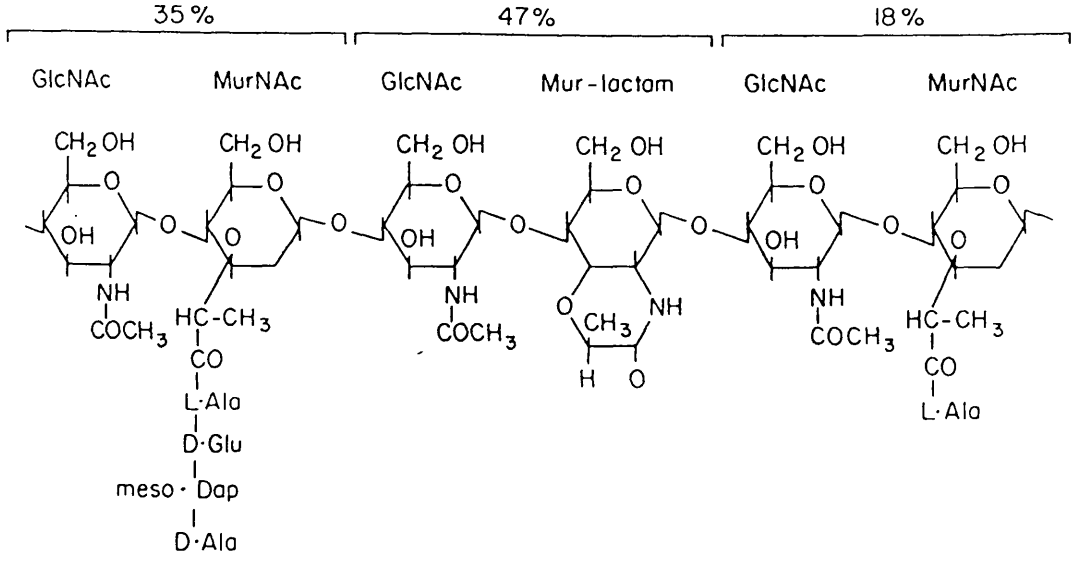
1.2.2 Cortex

The spore cortex is located between the core and the spore coats. The structure of the spore cortex appears to be constant from species to species. *B.stearothermophilus* cortex has a fibrous structure, observed in electron micrographs of thin sections [10]. During maturation, it becomes bonded to the spore coats and the incorporated mother cell cytoplasm by tertiary changes in structure. The inner layer (cortical membrane) develops into the cell wall of the emergent cell when cortex lysis occurs during germination. The first evidence of the structure of the cortex came from studies of "germination exudate", the muropeptides in the exudate arising from lysozyme degradation of the cortex during germination [11, 12]. The cortex is made up of peptidoglycan (muropeptide, murein). The structure is similar to, though not identical to,

the structure of vegetative cell peptidoglycan. Peptidoglycan consists of a polymer of alternating N-acetylglucosamine and N-acetylmuramic acid residues [13] (Figure 1.4). In spore peptidoglycan, 45-60% of the N-acetylmuramic acid residues form internal amides known as muramic lactams, leaving the amino acid residues with no free carboxyl groups. In a further 20% of the N-acetyl muramyl groups a single L-alanine is attached to their carboxyl groups. The peptide modifications to the general structure are thought to take place after the synthesis of the polymer. The average length of the glycan chains in the cortex of *Bacillus* spores is estimated to be from 80 - 100 saccharide units [15]. Although there are significant variations in the peptidoglycan of vegetative cell walls, the structure of cortical peptidoglycan is largely conserved between species [9]. Spore peptidoglycan does not appear to be associated with the usual cell wall polymers such as teichoic acid, teichuronic acid, and complex polysaccharides [14].

Figure 1.4. Chemical structure of spore peptidoglycan [25].

The relative frequency of each unit is shown as a percentage.



Diaminopimelic acid (DAP) is almost unique to spores, and is present in the spores of most species. The spore peptidoglycan is loosely cross-linked, about 80% of the DAP ε-amino groups in the cortex of *B.coagulans* being free [15], in contrast to most cell walls which are highly cross-linked. DAP is laid down in two phases during cortex formation. It is laid down early in the base layer that persists following germination. Late deposition of DAP is lost during degradation of the cortex [13]. The role of the cortex in heat resistance and dormancy of the spore is discussed later.

1.2.3 Spore coats

The spore coats consist largely of protein and mucopeptides, with DAP (α-ε-diaminopimelic acid) and hexosamine. In thermophilic bacteria, the DAP and hexosamine were generally found to be in the insoluble integument fractions. The spore coat consists of two components, the inner coat and the more distinctive, layered outer coat. Solubilisation of 80% of the total coat protein occurs when the inner coat of *B.cereus* spores is removed with thioglycollate extractions. This layer consists of cross-linked polypeptides, rich in disulphide linkages. The soluble fractions of spore coat structures of thermophilic bacilli, *B.coagulans* and *B.stearothermophilus* were shown to have higher concentrations of DPA and hexose compared to other bacilli [16]. The hexose content was apparently related to heat resistance properties of spores. The more insoluble outer layers form during a specific period in sporulation, correlated with rapid uptake of cysteine from the medium, or increased cysteine content of the coat layers. An increase in cysteine is found in the spore coats even if no cysteine is added to the medium. This cystine is exchanged with pre-existing protein sulphydryl or disulphide groups resulting in conformational change of the polypeptides.

Coat protein precursor synthesis begins when growth ceases, during early forespore development, several hours prior to the appearance of mature coat structures [17]. Completion of coat formation is a late event in sporulation, occuring at the same time as full refractility and heat resistance. An increase in cysteine is found in the spore coats even if no cystine is added to the medium. There are variations in the size and appearance of the coat layers in different species, but the general components are similar. Appearance of spore coats during sporulation correlates with the conversion from phase bright to phase dark spores under phase contrast optics.

Warth *et al* [16] studied the structure of spores of four bacilli, *B.cereus*, *B.subtilis*, *B.coagulans* and *B.stearothermophilus*. They found that the general morphological features were very similar. The inner coat appeared laminated in all species, but there were differences in the structure of the outer coat. These differences in structure may be related to their resistance properties.The outer and inner coats of spores were difficult to separate completely. *B.stearothermophilus* appears to have a thinner outer coat than other species, having a uniform, fine-grained appearance. The outer coat of other species, such as *B.subtilis* and *B.cereus*, show a loose network structure, whilst a very electron-opaque coat is found in *B.coagulans*. A higher proportion of insoluble material was obtained from the more heat-resistant spores. Spores of *B.stearothermophilus* have higher amounts of DPA and sugars in both their soluble and insoluble coat fractions, possibly related to greater heat resistance compared to other spores. The hexosamine and DPA found in coat fractions seems to be associated with residual cortex and cortical membranes [16].

The amino acid composition of spore coats is high in glycine, lysine, aspartate and glutamate and non-polar amino acids. The cysteine content of spore coats is five times that of vegetative cell protein, suggesting the possibility of intra- and intermolecular

bonding of the hydrophobic type, as well as production of intramolecular disulphide bridges and ion pairs. Large amounts of phosphorous are found in the coats of some species.

Spore coats are resistant to proteolytic enzymes and a wide variety of chemicals, due to the stability imparted to it by hydrophobic interactions and covalent cross-linkages. The coats have no significant role in heat or UV resistance. Inhibition of synthesis, removal of coat proteins, or disruption of coat structure by mutation, produces spores with near to normal heat and UV resistance which retain their calcium and DPA. Such spores differ in their response to lysozyme, octanol and germinants, suggesting a role as a protective barrier to the cortex [8].

Stelma *et al* [18] isolated mutants of *B.cereus* that had a slow response to germinants and were sensitive to lysozyme. The spores had normal heat resistance properties and cortex structure and DPA content were unaltered. The coat protein was synthesised and assembled into structural layers, but the coat layers were deposited in the cytoplasm rather than on the forespore membrane. It is thought that sites of deposition on the membrane were abnormal. Therefore, coat formation and maturation, and formation of the spore body can be regarded as independent processes [19]. The altered response to germinants suggested a major role of the spore coats in germination.

1.2.4 Exosporium

This is an outer membranous layer, which may be loosely or tightly fitted around the outside of the spore. This layer is present in *B.cereus*, but absent from *B.stearothermophilus*. Isolated exosporium of *B.cereus* contained 52% protein, 20% polysaccharide containing glucose, rhamnose, glucosamine and ribose, 12.5% neutral

lipid, 5% phospholipid and 3.8% ash [20]. The protein had a low methionine and cysteine content in contrast to coat protein. The exosporium is produced outside the OFSM after engulfment of the forespore has proceeded. It engulfs the forespore as the cortex accumulates, the coat layers forming inside the developing exosporium layer. The exosporium proteins are highly resistant to proteases, giving an additional protection to the interior of the spore [19].

1.3 Sporulation

1.3.1 Factors affecting sporulation

Sporulation in *Bacillus* species is induced when carbon, nitrogen, phosphate or sulphate is limiting in the growth medium. Properties of the spores produced will be dependent on which of these nutrients is depleted. Studies of *B.megaterium* [21] and *B.stearothermophilus* [22] have shown that spores produced in carbon-depleted medium germinated faster and to a greater degree than sulphur-depleted spores. The concentration of manganese in the sporulation medium also had an important effect on the germination of carbon-depleted spores. Increased manganese concentration in the sporulation medium led to an increase in the germination rate. Mn^{2+} ions act as a cofactor for 3-phosphoglycerate mutase, which provides energy to the germinating spore from stored 3-PGA [23].

Depletion of sulphur during sporulation appears to lead to incomplete coat formation, due to the abundance of sulphydryl bridges in coat proteins.. The spore coat layers are believed to be vital in germination, and thus the incomplete nature of the coats of sulphur-depleted spores seems to hinder germination. Heat activation in *B.cereus*, leading to an

increased germination rate, also caused an increase in the thiol groups in the coat proteins [24].

The conditions that are suitable for optimal cell growth may inhibit sporulation or lead to a low spore yield. This may be due to factors such as a high concentration of nutrients. During vegetative growth, conditions in the medium are constantly changing, therefore it is difficult to determine the effect of one factor in the medium, since different factors may be inter-dependent. For example, glucose concentration, pH and aeration rate are inter-dependent factors in the sporulation of *Bacillus licheniformis* [7]. Sporulating cells generally have a narrower range of tolerable pH values than the corresponding growing vegetative cells. Arginine has been reported to be important in the sporulation of *Clostridium botulinum* [7, 25]. Stationary phase cells of several species can undergo sporulation in a nutrient-free environment. This is termed "endotropic sporulation". Spore proteins are synthesised during sporulation, therefore in this type of sporulation, degradation of macromolecules to low molecular weight products serves as the only carbon and energy source, since there is no exogenous supply [26].

Temperature has an effect on vegetative growth, extent of sporulation, and the properties of spores. Elevated temperature during growth led to *Bacillus stearothermophilus* spores with reduced heat resistance properties [27], whilst in *Bacillus subtilis* and *Bacillus coagulans*, it led to an increase in the heat resistance [28]. An increase in the aeration rate is required for pre-sporulation and for the optimal sporulation of most bacilli, including *Bacillus stearothermophilus* [29].

Grossman and Losick [30] observed that sporulation could not be efficiently induced in cells grown at a low population density. However, sporulation was stimulated if cells grown at a very low population density were transferred to medium that had been

previously conditioned by cells grown at high density. Apparently a substance, possibly an oligopeptide, was produced by vegetative cells grown to a high density to stimulate them to sporulate.

1.3.2 Morphological changes

The morphological changes that occur during sporulation were first described by Young and Fitz-James [31, 32]. The ordered sequence of events beginning at the end of logarithmic growth and concluding with the production of a mature endospore has been divided into seven stages (I-VII) (Figure 1.5), based on electron microscopic observations of cell sections [7, 33]. The end of the logarithmic phase of growth is regarded as Stage 0. This is rapidly followed by the transformation of the disperse nuclear material of growing cells into an axial filament of condensed chromatin (Stage I). Further DNA synthesis does not appear to occur at this stage [31, 32].There is no qualitative difference between the DNA in spores and in vegetative cells, although the number of genomes per spore may be variable.

A septum begins to form at one end of the cell during Stage II. The nuclear material becomes separated into the two components, now referred to as the mother cell and forespore. The forespore cytoplasm occupies approximately 20% of the vegetative cell volume. The septum forms as a result of new macromolecular synthesis and is not due to rearrangement of the pre-existing vegetative cell membrane. In Stage III, the forespore protoplast is engulfed by outgrowth of the cytoplasmic membrane, resulting in two facing membrane surfaces adjacent to the forespore cytoplasm. These are known as the inner and outer forespore membranes. Peptidoglycan precursors are transported across the space between the membranes. Cortical peptidoglycan and the germ cell wall are synthesised during Stages III and IV [34]. The forespore increases in volume six fold in

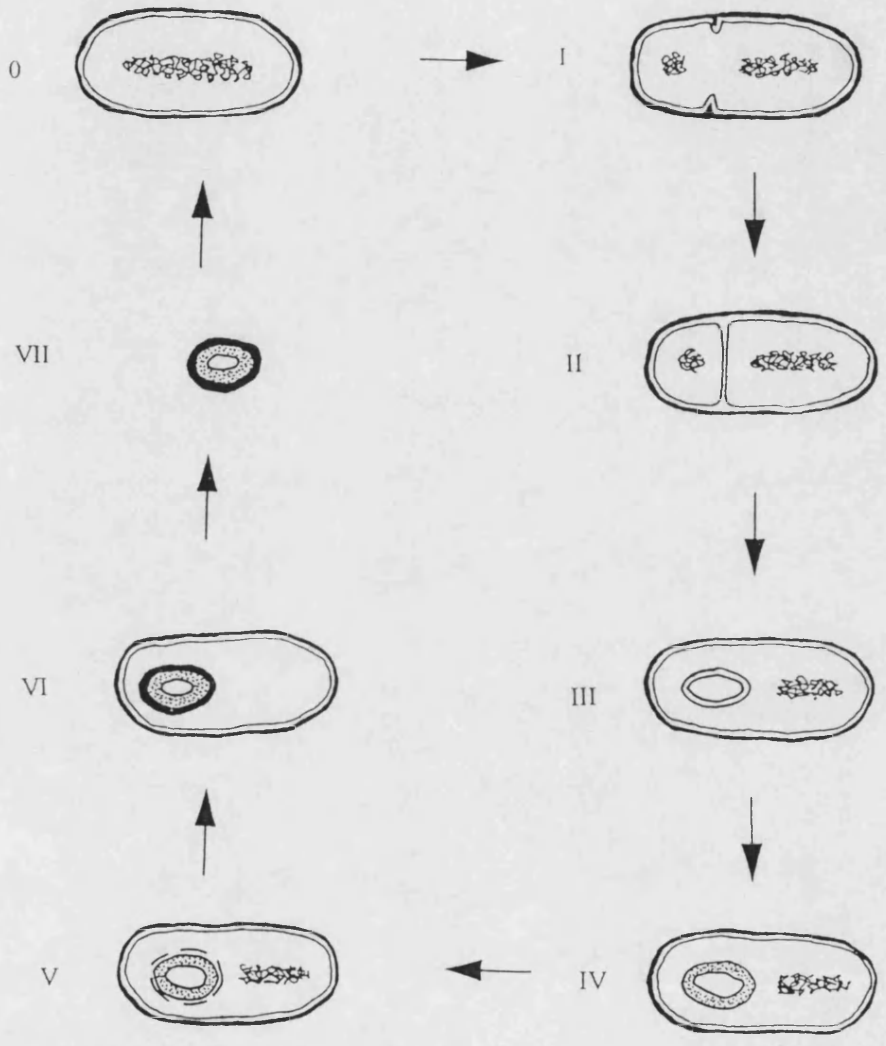
B.subtilis between Stages II and IV [34]. The forespore is not capable of maturation independently from the mother cell at this stage in its development. The sporulating cell reaches a stage of commitment, such that it can no longer withdraw from completing the formation of a spore prior to vegetative growth. Before this stage, if fresh medium were added, the cell would revert to vegetative growth. After this stage, addition of nutrients would lead only to a delay in sporulation.

During Stage IV and early Stage V, the forespore becomes partially refractile. This coincides with the commencement of cortex synthesis. During this period, formation of the spore coat layers and exosporium occurs.

It has been reported that the production of coat proteins is initiated shortly after the end of exponential growth and occurs throughout the early stages of sporulation, although the assembly and incorporation of cysteine occurs in Stage V [35]. Spore coat assembly appears to occur partly by self-assembly of proteins that have been synthesised at an earlier stage.

Synthesis of nine of the fourteen proteins that are components of the coat layers in *B.subtilis* starts in Stages II and III. The assembly of the coat layers markedly affects the germination and heat resistance properties of the resultant spore [36]. Mutants with altered or incomplete spore coat assembly which produce spores with altered resistance or germination properties have been studied [37]. The completion of Stage V is signalled by the end of coat assembly [38]. Resistance of *B.subtilis* spores to octanol and chloroform appears during Stage V, after the development of refractility and prior to the development of heat resistance [38]. Stage VI consists of a maturation process in which heat resistance develops and changes in the spore protoplast occur, giving it a more even, electron-dense appearance. This requires the continued synthesis or modification of the

Figure 1.5. Diagrammatic summary of the stages in the sporulation of *Bacillus* species.



0	Vegetative cell
I	Axial filament formation (pre-septation)
II	Formation of septum
III	Engulfment of forespore (encystment, envelopment)
IV	Cortex formation
V	Coat formation
VI	Maturation (development of refractility and heat resistance)
VII	Release of forespore

cortical peptidoglycan and the uptake of DPA and calcium. The coat becomes more dense and the spore core becomes dehydrated. The final stage (VII) involves liberation of the mature spore by autolysis of the mother cell.

1.3.3 Genetics of sporulation

Most of the regulatory genes determining the pattern of gene expression during sporulation have now been characterised. They are generally interspersed around the chromosome with genes having no role in sporulation. Initial genetic studies located loci involved in the control of sporulation. These genetic loci were designated *spo* genes. The designations *spo0*, *spoI*, *spoII*, *spoIII etc.*, indicated that mutations in these loci would allow progression of sporulation to stage 0, I, II, III respectively, but not beyond [39]. Several other categories of genes involved in sporulation have been characterised. Genes involved in germination were identified by *ger* mutations, and were initially thought to have no effect on sporulation. However, germination is now known to take place in the absence of *de novo* gene expression, therefore genes responsible for the germination response must be expressed during sporulation. The distinction between *spo* and *ger* designations is often arbitrary. For example, the *gerE* gene was first identified by its effect on germination, but is now known to encode a DNA-binding protein that regulates gene expression during the late stages of spore coat synthesis [40]. Some *spo* mutants produce spores with altered germination properties, such as *spoVIA* [41] and *spoVIB* [42]. Other sporulation-specific genes have been identified, such as those encoding sporulation associated enzymes, spore coat proteins and small acid-soluble proteins of the core [39].

A series of phosphotransfer reactions, termed a multi-component phosphorelay, has been postulated as the mechanism of initiation of sporulation in *B.subtilis* (Figure 1.6) [43].

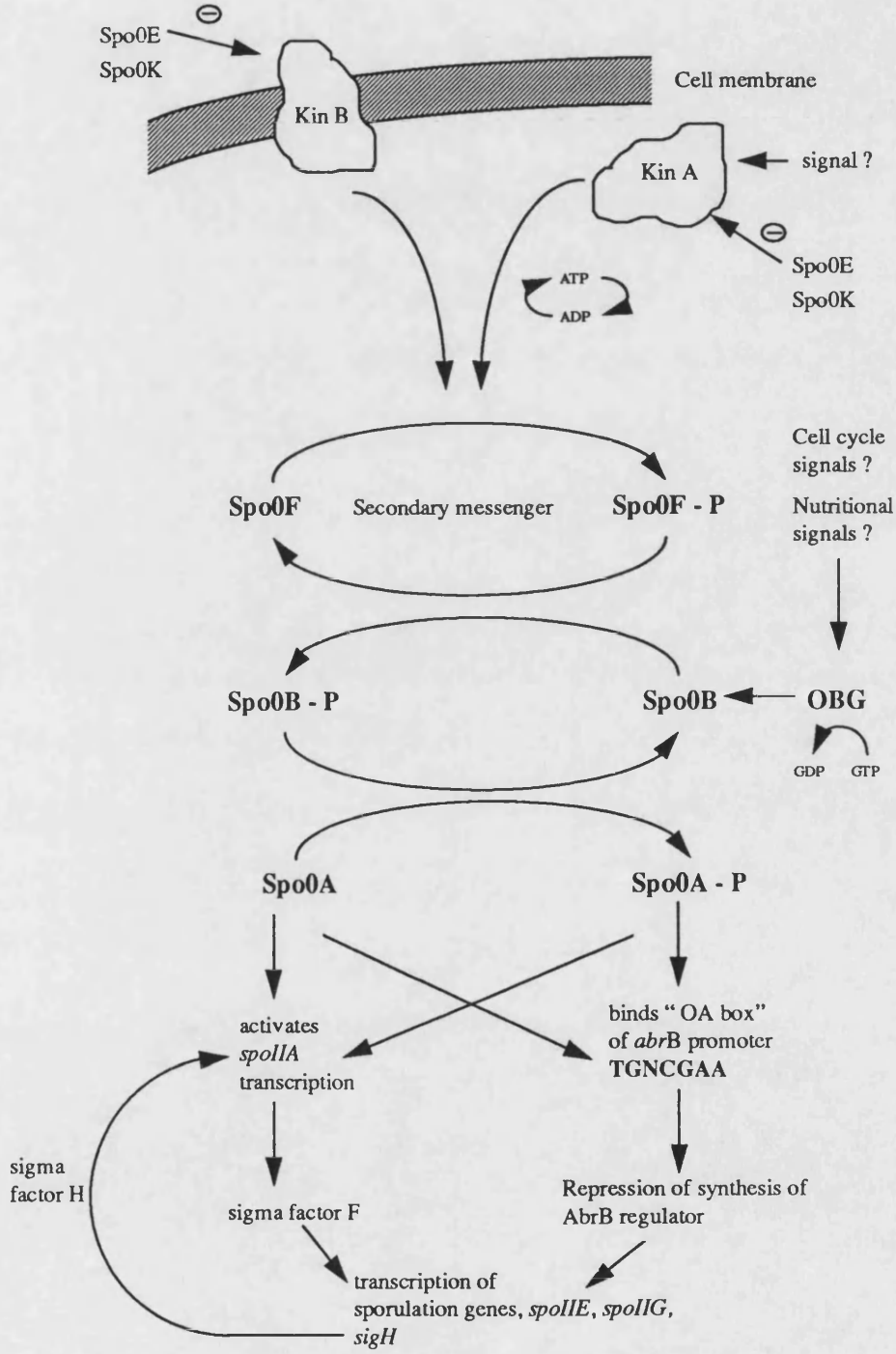
The sensitive nature of this type of control system is vital for a process such as sporulation, in which the expenditure of energy and the activation of hundreds of genes would be required should cell division cease and the sporulation cycle commence. Stage 0 sporulation mutants (*spo0*) that are permanently coupled to cell division and are unable to initiate sporulation have been employed as a tool in studying the control of the initiation of sporulation [44]. Several genes of these mutants have been mapped, *spo0A*, *spo0B*, *spo0E*, *spo0F* and *spo0H*. The key regulatory protein in this group is Spo0A. Spo0A acts as a negative regulator of the *abrB* gene, which codes for the AbrB regulator protein. AbrB binds to and prevents expression of promoters of genes that are activated at the end of stationary phase. This includes genes essential for sporulation, whose expression it prevents in vegetative conditions, even if normal controls give contradictory signals.

During vegetative growth, AbrB levels are high, repressing genes under its control. At the end of vegetative growth, Spo0A represses AbrB synthesis, inhibiting transcription, and the genes are released from the effects of AbrB. Spo0A is similar to other regulatory proteins of two-component regulatory systems common in bacteria [45].

The other component of such systems is a protein kinase whose function is to activate the response regulator by phosphorylation following environmental signals. Kinase A, the product of the *spoIIJ* gene, was found to phosphorylate almost exclusively Spo0F (and Spo0A at very low levels). In the absence of kinase A (*kinA* mutants), activation of the pathway is carried out by a membrane-bound kinase, kinase B, although sporulation is delayed and occurs at a low rate [46].

Figure 1.6. Diagrammatic representation of a model for the initiation of sporulation in *B.subtilis* by a multi-component phosphorelay.

Adapted from Burbulys *et al* (1991) [43].



Spo0B is a protein phosphotransferase that catalyses the transfer of phosphate from Spo0F-P to Spo0A. The Spo0B protein phosphotransferase transfers the phosphoryl group from the aspartate of one response regulator to the aspartate of another regulator. However, the phosphoryl group of Spo0B-P was freely transferable back to Spo0F. Spo0F acts as a second messenger, accumulating phosphate groups from several kinases as a response to environmental signals. Negative regulation of the pathway occurs via Spo0E, although the mechanism is unknown.

The Spo0B protein phosphotransferase is thought to be a control point of the pathway. The Spo0B operon consists of two genes, *spo0B* and *obg*. The *obg* codes for a GTP-binding protein [47], Obg, which stimulates growth in its GTP-bound form, and prevents sporulation by stimulating the phosphatase activity of Spo0B. Spo0A-P also activates transcription of *spoIIA* gene, which codes for a sigma factor, σ^F, which regulates transcription of sporulation-specific genes. Spo0A-P connects environmental, metabolic and cell cycle signals with the activation of transcription required for sporulation [43].

The forespore and mother cell compartments begin with identical genomes, but activation of different sets of genes leads to differentiation and their separate development into two distinct structures during sporulation [39]. After septation, σ^F activity is released specifically in the spore compartment where it activates at least 2 genes, *gpr* and *spoIIIG*. σ^F release requires the products of *spoIIE* and *spoIIIE* genes. Many genes are activated specifically in the mother cell compartment. Transcription in the two compartments is controlled by two sigma factors, σ^F and σ^E. Their activities are dependent on septum formation. Sigma factor, σ^E, is produced as an inactive precursor. Pro-σ^E is encoded by one of 2 genes in the *spoIIG* operon, the other gene in the operon, *spoIIGA,* is thought to code for the protease that activates σ^E. Similarly, the *spoIIA* operon encodes σ^F and the proteins that regulate its activity. Following septation, σ^F is released in the prespore,

under the influence of SpoIIAA and SpoIIAB proteins. This results in transcription of a gene whose product acts across the spore septum to trigger pro-σ^E in the mother cell [48, 49]. Different programs of gene expression are embarked upon by the mother cell and forespore due to the compartmentalised activities of σ^E and σ^F.

Engulfment of the forespore might involve a specific protein that mediates membrane fusion, and membrane phospholipid synthesis. Genes dependent on σ^E are required to initiate engulfment, septal peptidoglycan hydrolysis, and fusion of the septal membranes to complete the engulfment of the forespore

Sigma factor E, σ^E, is thought to be regulated by SpoIIID, which divides the σ^E genes into 3 temporal classes. The *spoIIID* gene expression is delayed by 10-20 minutes compared to other σ^E-dependent genes, since full expression of this gene requires its own product. Therefore genes requiring SpoIIID are delayed in their expression. However, genes that are repressed by SpoIIID are transcribed as soon as σ^E appears, but expression is temporary since they are shut down as soon as enough SpoIIID has been produced. This mechanism allows the timing and extent of expression of these genes to be finely controlled. Sigma factor F, σ^F, is only known to regulate transcription of two genes in the prespore, *gpr* and *spoIIIG*, in contrast to early mother cell development in which more than 10 genes are known to be regulated by σ^E [39].

After the completion of engulfment of the forespore (approximately 2 hours after initiation of sporulation), synthesis of σ^G, the product of *spoIIIG*, begins. This regulates the genes of the σ^G regulon, which vary significantly in their effects. Activation of σ^G appears to be related to completion of forespore engulfment. At least six σ^G-controlled genes encode small acid-soluble proteins (SASPs) found in the spore core. All SASPs have a short conserved sequence recognised by a specific protease encoded by the *gpr*

gene. The action of the protease must be controlled in order that SASP degradation occurs only after germination has commenced. The protease is synthesised as an inactive 46kDa precursor, which is cleaved to a 41kDa form late in sporulation, and to a 40kDa form during germination. Cleavage of the SASPs during germination initiates their complete degradation, releasing a source of amino acids for synthesis during outgrowth [50]. Some SASPs are bound to DNA in the spore nucleoid, causing an increase in negative superhelicity of the DNA, contributing to UV resistance and possibly heat resistance of spores.

A fourth sporulation-specific sigma factor, σ^K, controls the final stages of sporulation, regulating genes that encode synthesis and assembly of the cortex and coat, and those that lead to the final maturation and release of the spore [39]. The *sigK* gene promoter is activated by σ^E and accumulation of SpoIIID. Further transcription is regulated by σ^K itself. Sigma factor, σ^K, is produced as an inactive precursor, which is activated by proteolytic cleavage of a 20 amino acid residue sequence. Processing of σ^K is under the control of σ^G and several genes of the σ^E operon.

Dipicolinic acid (DPA) synthesis, which occurs specifically in the mother cell at a late stage of sporulation, appears to be dependent on σ^K. DPA is actively transported into the prespore, and Ca^{2+} enters by facilitated diffusion. Several *spo* mutants synthesise but do not incorporate DPA. Two other genes, *spoVK* and *spoVA* are postulated to have a role in DPA or Ca^{2+} transport. The *spoVK* gene encodes a single protein with an ATP-binding site, possibly involved in ATP hydrolysis to drive Ca^{2+} or DPA uptake into the prespore. Mutations in *spoVA* abolish heat resistance, but maintain partial resistance to lysozyme and organic solvents, suggesting an intact spore coat. The SpoVA proteins are likely to form a membrane-complex, possibly related to Ca^{2+} or DPA transport [51].

Spore cortex synthesis is likely to be due largely to the mother cell. It is similar in structure to vegetative cell peptidoglycan, but spore-specific proteins are involved in its modification. Genes of the σ^E and σ^K regulons are thought to be involved in regulation of cortex synthesis [39]. Several genes, *spoVD*, *spoVE*, *spoVB*, *gerJ* and *gerM*, have been implicated in cortex synthesis.

The spore coat consists of at least 12 different proteins assembled on the outer face of the outer prespore membrane. The order of synthesis and assembly of proteins does not necessarily coincide; self-assembly of pre-formed proteins is important in coat formation [52]. The GerE DNA-binding protein negatively regulates *cotA* and *cotT* genes and positively regulates *cotB*, *cotC* and *cotD* genes, controlling the timing and expression of these genes involved in spore coat synthesis. Mutations of most *cot* genes have little effect on sporulation or germination. Mutants in *cotE* have normal refractility but are lysozyme sensitive and have minor changes in germination properties. Such spores are deficient in CotE, but also other proteins that are synthesised and assembled in late sporulation, *eg.* products of *cotA*, *cotB* and *cotC* genes. It is thought that CotE is important as a basement protein on which other outer coat proteins assemble [53]. CotE has sequence homology with peroxidases. Such cross-linking activity could be important in covalent assembly of the outer coat. Proteins encoded by *cotD* and *cotT* genes are assembled in the inner spore coat. Mutations of these genes lead to minor alterations in germination, suggesting a role of the inner coat in germination. Mutations of the *spoIVA* gene lead to production of spore coat material, but errroneous deposition of the material as masses in the cytoplasm occurs, rather than on the surface of the prespore membrane [39].

1.4 Activation, Germination and Outgrowth of Spores

There are three processes involved in the transformation of a dormant bacterial spore into a vegetative cell ; activation, germination and outgrowth. Each process involves distinct biochemical changes within the spore. Dormancy is defined as the inability of spores to germinate. Activation is the conditioning of the spore to germinate, and is usually a reversible process, the spore essentially retaining its original features. Germination is an irreversible process in which the spore loses all typical spore characteristics, but remains distinct from vegetative cells in composition. Outgrowth is the process of biological growth and differentiation, leading to conversion of the germinated spore into a vegetative cell [54].

1.4.1 Activation

Activation is thought to involve a change in the configuration of macromolecules, and essentially no metabolism. Germination involves the breakdown and excretion of compounds into the medium, and the initiation of spore metabolism. The most common and efficient method of activation is exposure to sub-lethal heat. Exposure to low pH (1 - 1.5), thiol compounds and strong oxidising compounds are also effective in activating spores. Activated spores retain their resistance to heat and radiation, non-stainability and refractility. Spores from freshly-harvested cultures germinate only very slowly, with a lag period preceding germination. The germination in this situation is asynchronous and incomplete, many ungerminated spores remaining in the suspension. Activation decreases the lag time and increases the rate of germination. The degree of dormancy of individual spores in a spore population is variable, and therefore not all spores germinate even after activation, *ie.* there is phenotypic heterogeneity in any population of spores [54].

The fact that specific agents are needed for spore germination was first demonstrated by Hills [55]. It was found that glucose, adenosine, inosine, L-alanine and some other amino acids, alone or in combination, were capable of causing 90% germination in several strains of bacilli. Activation of these spores altered their requirements for subsequent germination, either reducing the concentration of glucose or L-alanine required to give the same degree of germination, or allowing germination in a more restrictive medium than needed by unactivated spores [56, 57, 58]. No metabolic activity was measurable in dormant spores, but activation induced activity [59]. Activation of glucose metabolism by heat was reversible during storage. Concentrations of L-alanine and adenosine were able to induce glucose oxidation without initiating germination [60]. Activity of other enzyme systems was also enhanced by heat-activation. For example, 25% of the activity of NAD-linked glucose dehydrogenase was found in untreated compared to heat-activated spores [61]. There was also an increase in acetokinase and CoA-kinase actvities in heat-activated spores [62]. Activation is usually measured by the kinetics of subsequent germination. Germination is measured either by decrease in optical density, or by direct microscopic examination of the loss of refractility and the phase darkening of spores. The time and temperature of activation which is required is species and strain-dependent. *B.stearothermophilus* and other thermophiles may require 105 - 115°C for optimal activation [63]. Lower temperatures, 80-100°C, have been shown to cause heat-induced dormancy. This work also showed that these spores could be reactivated by exposure to higher temperatures. The sporulation temperature of the bacteria has a profound influence on the activation and germination requirements. Powell and Hunter [56] reported that heat activation had to take place in an aqueous environment, and that it was not possible to activate dry spores. They also found that activation was pH-dependent, and that a low pH environment led to efficient activation. The composition of the heating medium was also found to be important in heat activation, *eg.* heavy metals, surface active agents, salts. Activation of *B.stearothermophilus* spores usually results from

exposure of the spores to 115°C for several minutes [64]. This activation was shown to be a reversible process and that deactivation could occur spontaneously on storage or could be induced by chemical means. Maintenance of the dormant state is likely to be contributed to by cysteine-rich coat proteins, stabilised by disulphide linkages. Reduction of these bonds during activation, which would lead to partial unfolding of these proteins, and could be responsible for increased permeability and revealing active sites of enzymes or increased accessibility of substrates. Reversibility of the activation could be due to the reoxidation of sulphydryl groups and reformation of disulphide bridges.

Agents other than heat, such as low pH, reducing agents, calcium dipicolinate, ionising radiation, and the process of ageing can also cause spore activation. Ageing can be seen as having the same effect as heat activation, but involving long term exposure of spores to low, stable temperatures. Increased permeability of spores and change in structure of spore macromolecules have been suggested as mechanisms for activation. The spore coat has been hypothesised as the first target site in the activation process [65, 66, 67]. Denaturation of proteins in the outer spore coat and the action of disulphide-reducing agents, *eg*. mercaptoethanol, in the spore coat led workers to believe that the spore coat is the first target in heat activation [65, 66, 67]. It is likely that activation is not primarily caused by a metabolic event, but is more likely to be a physicochemical effect, such as a change in the tertiary structure of spore macromolecules, since the abrupt thermodynamics correlate well with heat denaturation of proteins.

1.4.2 Germination

1.4.2.1 Factors affecting germination

Germination is the term used to describe the conversion of a resistant, dormant spore into a sensitive and metabolically-active form [68]. This can occur in a very short time period, with little utilisation of exogenous substances. During germination, spores lose their resistance to heat, pressure, UV and ionising radiation, antibiotics, chemicals and low pH. Germination can be induced by Ca-DPA, L-alanine, purine riboside, various sugars, lysozyme, hydrostatic pressure and other mechanical processes. It has been suggested that calcium and DPA act together to stabilise proteins and nucleic acids. It may be that the resistance of spores is determined by the nature of DPA-metal-protein bonds, the rupture of which leads to the breaking of dormancy. In some species, heat activation is necessary for rapid germination. The loss of heat resistance was the first criterion used to measure germination, the reduction of heat resistance being in the order of 10^3 to 10^6-fold. Germination is a degradative process which does not involve a significant level of synthesis of new macromolecules. The increase in enzyme activity seen during germination is due to activation of existing enzymes, rather than new synthesis.A great increase in respiratory activity occurs during spore germination. Some enzymes are detectable in ungerminated spores, eg. enzymes active against germinants, such as alanine racemase, alanine dehydrogenase and nucleoside phosphorylase.

During germination, excretion of materials up to 30% of the dry weight of the spore occurs. This exudate comprises calcium, an equimolar amount of DPA, depolymerised peptidoglycan, small amounts of amino acids and small peptides and proteins. Calcium is strongly bound in the spore but is rapidly lost during germination. Some of this calcium is exchangeable with other cations. It is known that in the vegetative cell, calcium is

strongly bound by peptidoglycan, therefore it is probable that depolymerisation of the cortex peptidoglcan causes release of calcium from the spore. The principle change in spore structure during germination is the near complete loss of the cortex structure of some organisms, eg. *B. cereus*, *B. anthracis*, *B. megaterium*. Sometimes the loss of the cortex is preceded by an initial swelling or a change in the cortex texture, to become spongy or fibrillar. The observed fall in optical density during germination is mostly due to the excretion and solubilisation of dry matter from the germinating spores. Spores change from phase bright to dark, as viewed by phase contrast optics. This change correlates with the loss of heat resistance. Phase darkening is caused by the effects of excretion of dry matter, swelling, and possibly the redistribution of water within the spore. Depolymerisation of cortex peptidoglycan could reduce the pressure on the spore core and lead to local hydration of this area.

L-alanine has been shown to act as a germinant in many bacilli, including several strains of *B.stearothermophilus* [69, 70, 71]. Watabe *et al* [77] showed that L-alanine was initially incorporated into spores of *Bacillus thioaminolyticus* during germination, released into the germination medium, and then reincorporated into the spores after 2 minutes at 37°C. A correlation existed between the contact time of the spores with L-alanine and the initiation of germination. A commitment time for the initiation of germination was between 2 and 3 minutes. Incubation with ^{14}C-L-alanine for 2 minutes or less did not induce subsequent germination in the absence of L-alanine. Other amino acids were capable of substitution for L-alanine.

Some spores are also known to germinate in response to other amino acids and their analogues. Heat-activated spores are often able to germinate in a wider variety of amino acids than unactivated spores. For example, untreated *B.megaterium* QM B1551 spores germinated in L-alanine, whereas heat-activated spores were also able to germinate in L-

cysteine, L-leucine, DL-isoleucine, DL-methionine, DL-nor-leucine, L-proline and L-valine. Germination is often incomplete if a single germinant is used. This phenomenom is termed fractional germination and may be due to changes in the germination medium throughout the process of germination.

The sequence of events during germination of *B.cereus* spores occured as follows; loss of heat resistance, release of DPA, onset of stainability, phase darkening of spores and a fall in the optical density of the spore population [72, 73]. It is thought that changes during germination arise from the hydrolysis of cortex peptidoglycan, followed by diffusion of peptidoglycan fragments and low molecular weight components such as calcium and DPA. It is possible that initial hydrolysis is able to destroy properties which maintain heat resistance, but only after subsequent hydrolysis are fragments able to diffuse out of the spore [74, 75, 76].

The outer layer of the spore coat seems to be a loosely-packed structure to allow the penetration of low molecular weight substances, such as alanine and glucose, into the inner layer [78]. Therefore it is suggested that the outer coat does not actually participate in the germination process, and that the initial site of action of germinants is in the inner coat layers. Change in permeability of spores during germination is due to alteration in coat structure. L-alanine was incorporated into an inner coat layer during the first stages of germination, by attachment to the inner layer or engulfment into the fibrillar layer.

Yasuda and Tochikubo [76] investigated the relationship between glucose and L- and D-alanine in initiation of germination. Glucose alone (up to 0.1M concentration) had only a small effect on initiation of germination. However, in the presence of L-alanine-induced germination, glucose was able to increase the binding affinity of L-alanine to sites within the spore and to increase the germination rate. A number of glucose analogues also had a

similar effect, suggesting that a specific binding site with structural requirements could be present on the spore. These effects can either be measured by loss of heat resistance (early event in germination), or by a decrease in optical density (late germination event). The first irreversible step of the trigger reaction, loss of heat resistance, seems to represent the commitment of the spore to germination [79]. D-alanine was found to be a competitive inhibitor of L-alanine-stimulated germination. Therefore it is likely that the binding sites within the spore for D- and L-alanine and glucose are arranged close together. Glucose had little effect on binding of D-alanine, indicating separate binding sites for these compounds.

Heat treatment of *B.subtilis* spores at 65°C for 30 minutes caused an increase in the germination rate compared to L-alanine and glucose, but it reduced the co-operative effect of glucose on L-alanine binding, without influencing the binding affinity of L-alanine or D-alanine [80]. Glucose dehydrogenase (GDH) was studied in resting spores, dormant spores and vegetative cells of *Bacillus subtilis* [81]. GDH exists in resting spores as a monomer and is activated during germination and outgrowth to become an active dimeric form, or the enzyme could be in the form of a dimer and tetramer, respectively. During germination, particulate NADH oxidase activity increases, suggesting the possibility that glucose is oxidised to gluconate by GDH and NAD, and that NADH produced is reoxidised by particulate NADH oxidase for production of ATP in the germinating spore and outgrowing cell. Germinated spores contain only a small quantity of cytochrome in spite of high particulate NADH oxidase activity. The generation of ATP, in that situation would occur only between NADH and the flavoprotein-CoQ complex until completion of the cytochrome system [82].

Dormant spores contain enzymes required for macromolecular synthesis and energy metabolism, yet the are essentially metabolically inactive. Generally spores contain less

than 10pmol/mg of ATP. Upon germination, the ATP level is rapidly increased to more than 3nmol/mg after 10 minutes by initiation of glucose catabolism [83]. Setlow and Kornberg [84] proposed the role of the glycolytic pathway in ATP provision. In this case, ATP for the initial phosphorylation reaction would need to be provided, presumably by anaerobic metabolism of 3-phosphoglyceric acid (3PGA). Sano *et al* [83] proposed an independent pathway of glucose metabolism, specific to spores, which did not require prior phosphorylation by ATP produced via 3PGA. ATP was provided by the aerobic oxidation of NADH, primarily produced by GDH, with glucose as a substrate, since dormant spores contain virtually no ATP. It is possible that phosphorylation by ATP derived from 3PGA is responsible for metabolism of other carbon sources.

Strauss proposed an important role of GDH in germination, finding that GDH-less spores of *B.subtilis* could not germinate well on glucose, but had a normal germination response to L-alanine [85]. Glucose is metabolised mainly via the glycolytic and pentose phosphate pathways in growing cells of *Bacillus subtilis*, and is also oxidised to gluconate without phosphorylation in spores, using GDH. The gene coding for GDH is expressed exclusively in the forespore under the influence of σ^G. It is unusual that GDH is synthesised at this stage, since sporulation is generally triggered after glucose depletion. It is therefore likely that the GDH may have an important function in germination [86]. Irie et al [87], however, isolated a *gerK* mutant which had lost the germination response to glucose, but had normal GDH activity. The *gdh* and *gerK* loci appear to be closely linked, and it possible that the mutant isolated by Strauss had other genetic defects resulting from the mutagenesis method. In contrast, Sano *et al* [86] found that *gdh* mutants germinated normally on glucose. In addition, spores which could not catabolise glucose due to three defective enzymes, triggered germination normally on glucose. Therefore it is unlikely that GDH or any other catabolic pathway has a role in the triggering of germination. The role of GDH is likely to be late in germination, producing

energy for germinating spores and outgrowing cells. In *B.megaterium*, glucose metabolism is not needed to trigger germination [84, 88], therefore GDH is not a universal prerequisite for germination, but fills the demand for ATP in germinating spores.

The germinant action of ribosides was first reported by Hills [69, 70], who reported activity of adenosine in germinating some *Bacillus* spores. He found that inosine, the result of the deamination of adenosine, is generally more active. Spores of *B.stearothermophilus*, *B.subtilis*, *B.coagulans* and *Clostridium* species are unresponsive to ribosides. In most cases it is necessary to have a combination of germinants for complete germination, usually L-alanine and inosine (or adenosine). Glucose may enhance germination of some spores, but can usually be replaced by other germinants.

A major contributing factor in the rate of germination is the type of medium used to induce sporulation. Sulphur-depleted spores of *B.megaterium* and *B.stearothermophilus* appear to germinate more slowly and to a lesser extent than carbon-depleted spores [21, 22]. Depletion of sulphur during sporulation may lead to changes in spore coat proteins (cysteine-rich), such as hydrolysis during germination, as well as possible interactions with germinants. Spores of *B.stearothermophilus* require glucose for germination. Exposure of *B.stearothermophilus* spores to 80°C for 10 minutes enhanced dormancy of spores, which could not be reversed by L-alanine or glucose. The rate and extent of germination increased in direct proportion to the intrasporal manganese concentration, which was directly related to the initial concentrations of this ion in the sporulation medium. Free Mn^{2+} ion is the preferred cofactor for the 3PGA mutase enzyme. Exogenous manganese, however, exerts an inhibitory effect on the germination of some species [22].

Surfactants, *eg.* cationic surfactants such as *n*-dodecylamine (laurylamine) have been shown to induce changes that mimic physiological germination, *ie.* loss of resistance to heat and chemicals, reduction of the optical density of spore suspensions, excretion of calcium, DPA and muropeptides, although the surfactant would kill the newly germinating spores if left in contact after the initial activation had occured.

1.4.2.2 Genetic control of germination

Analysis of mutants defective in spore germination (*ger* mutants) identified some genes that were necessary in the process [89, 90]. Germinants are thought to act by activating a series of changes in spore structure, brought about by hydrolysis of various spore components. Two mechanisms of stimulation of the germination response have been identified in *B.subtilis*. Germination can either be stimulated by L-alanine and its analogues and / or by the AGFK pathway (asparagine + glucose + fructose + potassium ions).

Mutants have been isolated that affect germination by L-alanine, but show no change in germination by the AGFK pathway. At least three operons controlled by σ^G are essential for germination. They encode systems that detect envirnmental conditions and regulate the germination mechanism. The *gerA* operon consists of 3 genes, *gerAA*, *gerAB*, and *gerAC*. The 3 protein products probably form a membrane complex in the inner prespore membrane. Mutations of any of these genes cause a defect in the germination response to L-alanine, but have no change in response to AGFK. The phenotypes of *gerA* mutations are consistent with this locus encoding the receptor for L-alanine within the spore [91]. GerAA (53.5kDa) has a membrane-embedded domain and two outer hydrophilic domains. Mutations of *gerA38* or *gerA44* require higher concentrations of germinant for successful germination. These mutations map in the *gerAB* gene, suggesting that the

GerAB protein (41.3kDa) may represent the primary L-alanine binding site. GerAC (42.4kDa) protein is hydrophilic, but contains the signal sequence of a pre-lipoprotein, suggesting an outer membrane location. These *gerA* genes are expressed at very low levels in the forespore, under the control of σ^G. It is likely that the GerA proteins remain at or near the surface of the spore inner membrane [89].

Mutations in the *gerB* locus cause a defect in the germination response to AGFK. A similar protein is encoded by *spoVAK*. A *gerD* germination gene is required for later stages of AGFK germination, and L-alanine germination under certain conditions. Another locus, *gerC*, is concerned with vegetative growth as well as germination. It encodes an operon of 3 genes. The product of the third gene, *gerCC*, is homologous to one sub-unit of an enzyme that condenses 2 geranyl pyrophosphate molecules to form a C_{40} terpinoid. This enzyme is apparently essential for normal growth of *B.subtilis*, presumably in membrane function, since terpinoids are thought to act as membrane stabilisers [92]. Mutants of *gerB* and *gerK* have normal L-alanine-stimulated germination, but defective AGFK germination. The *gerB* operon encodes 3 similar membrane proteins to GerA. Mutations in *fruB*, which codes for a fructose-1-phosphokinase enzyme, block the contribution of fructose to AGFK germination, suggesting metabolism of fructose as being important in triggering of germination. Two groups of germination mutants affect germination by both types of germinant. Mutations at the *gerD* and *gerF* loci cause slow germination in response to L-alanine and no germination response to AGFK. The *gerD* gene encodes a 21.1kDa hydrophilic protein, but contains a signal sequence. The *gerD* gene promoter is σ^G-dependent, and is expressed in the forespore. GerD is probably exported from the forespore and is found either free in the cortex or still associated with the outer membrane surface [89]. The products of the *gerD* and *gerF* genes are both essential for the early stages of spore germination, before loss of heat resistance. Other genes known to affect stages in the sporulation process, such as genes involved in coat

and cortex synthesis, also affect subsequent spore germination. All the genes responsible for the germination response must be expressed during sporulation, since no additional gene expression occurs during germination itself.

1.4.3 Outgrowth

Outgrowth is the development of a fully-functional vegetative cell from a germinated spore. The first event in outgrowth is the uptake of water and nutrients, and swelling of the spore. Synthesis of macromolecules, assembly of the cell wall, and other characteristic structures rapidly follow. The initial events during outgrowth depend on enzymes pre-existing in the dormant spore. Synthesis of enzymes that are absent or present in low amounts in the spore, is needed in the later stages. The process of outgrowth is asynchronous, since the germination of individual spores is completed at different times. The spore coat is shed intact, or may become fragmented during outgrowth [93]. Small-celled species, such as *B.subtilis*, appear to shed a firm coat which remains intact, whereas large-celled species seem to have a loosely packed coat which is absorbed or dissolved [94]. Synthesis of mRNA begins immediately after germination and proceeds during swelling and elongation of the cell. The development of enzymes of the tricarboxylic acid pathway occurs during outgrowth, the rate of synthesis being influenced by the composition of the medium. It seems that the pre-existing peptidoglycan structure of the cortex persists during outgrowth, and may provide the foundation of the cell wall of the newly-forming cell. The inner spore coat or the inner layer of the spore cortex is thought to develop into the emerging cell wall [95]. The synthesis of many enzymes begins at a specific time after initiation of outgrowth in *B.cereus* strain T spores, such as α-glucosidase, L-alanine dehydrogenase and alkaline phosphatase [4/1]. Many such enzymes are required to satisfy energy requirements of the outgrowing cell.

For spore outgrowth to occur, exogenous nutrients are required. Re-sporulation may occur without normal vegetative growth and division preceding sporulation, if nutrient depletion occurs. This is known as "microcycle sporulation" [96]. Some organisms can outgrow in simple, defined media, whereas others require complex nutrients. The nutrient requirement for outgrowing cells is greater than for fully-matured vegetative cells. Requirements of different species for exogenous sources of carbon, nitrogen, phosphorous and sulphur have been reported, since endogenous supplies appear not to be utilised [95]. Certain species require exogenous sulphur for outgrowth, since the sulphur derived from the breakdown of the spore coats appears to be non-utilisable. *B.subtilis* can effect outgrowth in the absence of added sulphur, and therefore must have an endogenous source [97]. Some spores require the addition of certain amino acids for outgrowth. For example, the minimum amino acid requirements for outgrowth of *B.stearothermophilus* spores at 55°C were isoleucine, leucine, valine, methionine, histidine, arginine (and also thiamin, biotin and nicotinic acid were needed) [95]. Certain metal ions, eg. Mn^{2+} were also required for outgrowth of some spores. Temperature and pH requirements for outgrowth may differ from the optimum germination conditions.

1.5 Spore heat resistance

1.5.1 General mechanisms of spore heat resistance

There is a great difference in the heat resistance properties of vegetative cells and spores of the same species of bacteria. Vegetative cells of organisms of the genera *Bacillus* and *Clostridium* are usually destroyed after less than 1 minute at 60°C, whereas the spores of the same species could survive many minutes at 121°C. Many of the molecules in the spore are similar or identical to those found in vegetative cells. Proteins are the most

important potentially labile components which must be stabilised in order for the spores to survive high temperatures.

There are three mechanisms interacting in heat resistance. Thermophiles generally produce the most heat resistance spores, psychrophiles generally produce the least resistant spores. This is termed inherent heat resistance. Most water within spores is associated with the cortex and coat, whilst the core is relatively dehydrated. The extent of core dehydration correlates well with heat resistance, although there are other contributory factors [98]. Bender and Marquis [98] used controlled acid titration to demineralise spores, then selectively remineralise them, without loss of viability of the spore population. Remineralisation of spores resulted in increased heat resistance, the calcium forms being approximately equivalent to the native forms. For restoring heat resistance of demineralised *B.stearothermophilus* spores, Na was the least effective salt, Mn was almost as effective as calcium, whereas Mg and K gave intermediate results.

Changes in mineralisation brought about changes in the hydration of the protoplast. The water content of spore protoplasts varied between 28% and 57%, in the *Bacillus* species studied, having a sharp cut-off at the upper and lower limits. It it probable that the lower limit of water content corresponded to remaining water being held in the bound state. Much greater pressure would be required to remove this bound water, and other mechanisms would be required to contribute to even greater heat resistance [99]. Beaman *et al* [100, 101] reported an exponential increase in the heat resistance, correlated with a decrease in the volume ratio of protoplast / sporoplast (sporoplast describing all structures bounded by the pericortex membrane, beneath the inner coat), due to relative shrinkage of the protoplast due to its dehydration.

Heat resistance of *Bacillus* spores was shown to increase with increasing optimum sporulation temperature [99]. Within a particular species, sporulation at higher temperatures led to greater heat resistances, *eg*. spores of *B.stearothermophilus* ATCC 7953 produced at 45, 60 and 75°C had D_{100} values of 30, 238, and 311 minutes, respectively [99]. This thermal adaptation mechanism affected the water content of the spore protoplasts.

Dehydration can be maintained by 4 possible mechanisms : the existence of a water impermeable barrier; the constituents of the protoplast having a low affinity for water; the system is in equilibrium with water vapour at less than 100% relative humidity, or a solution of water activity (a_w) less than 1; a pressure differential existing between the system and one at high a_w [23]. It has been demonstrated that the water impermeability barrier does not exist [101]. The second two mechanisms are not applicable for spores. Therefore, the existence of a pressure differential between the interior of the spore and the external environment is necessary for the maintenance of a partially dehydrated core. Several hypotheses have been suggested for the generation of appropriate pressure within the spore [98, 101, 102]. It has been reported that hydrolysis of the cortex with lysozyme causes loss of heat resistance, and blocking cortex formation prevents the development of heat resistance. The expanded cortex theory depends on the swelling of the cortex, which when restrained by the spore coats, produces a pressure on the core. This mechanism is not consistent with the observation that extraction of a large proportion of the coat material has little effect on heat resistance. The anisotropic cortex hypothesis states that pressure is applied in the radial direction only, with no outward expansion, with each layer transmitting its pressure to the core by compression of the layers beneath. Intermediate layers are in tension parallel to the surface, but under compression in the radial direction. For sufficient tension to be generated, the spore peptidoglycan must be more tightly packed than was originally thought. During sporulation, successive layers of

the cortex are assembled and the tension generated, then further layers added, which along with the Ca-DPA uptake into the core, is responsible for the dehydration of the core that is required for heat resistance [23]. The dehydration effect results from a tightly-packed matrix of peptidoglycan in the cortex which causes a dehydrated state to exist in the spore core, thus stabilising molecules and structures within the spore. Partly activated spores were found to be much more heat resistant than dormant spores. The increased heat resistance following heat shock could be explained by expansion of the cortex against the intact coat, resulting in reduced hydration of the core [100].

Table 1.1. Heat resistance and dipicolinic acid content of spores of Bacillus species (13)

Organism	D_{100} *(min)*	*% dry weight of DPA*	*Ca / DPA ratio*
Bacillus megaterium	2.10	8.80	0.76
Bacillus cereus	14.2	6.14	1.47
Bacillus coagulans	270	10.42	0.94
Bacillus stearothermophilus	714	13.55	0.96

The decimal reduction time (D_{100}) is the time required for a 90% (1 log cycle) decrease in the number of viable spores at 100°C.

Mineralisation is thought to play a critical role in heat resistance. This is largely the incorporation of large amounts of calcium (contributing up to 3% of the dry weight of the spore), but also other minerals, during Stage IV of the sporulation process. It is thought that manganese has an approximately equivalent contribution to heat resistance as does calcium. During the sporulation process, DPA is produced and accumulates in the forespore. DPA mainly exists as the Ca-DPA chelate, although it can also combine with

other metal ions. The specific role of DPA in heat resistance is unclear. Spores with high levels of DPA generally have high heat resistance (Table 1.1), although DPA-less mutants have been shown to be heat resistant. It is thought that incorporation of DPA into the core contributes to the displacement of water, and thus dehydration of the core [100].

It is thought that DPA does not have a unique role, but that other low molecular weight compounds can exert a similar effect. Any other factors that lead to stabilisation of macromolecules in the spore core, such as adsorption onto solids, will increase the thermoresistance of the spore.

The presence of certain media components at the time of sporulation has been reported to affect the heat resistance of the spores produced. Generally, divalent cations, especially Ca^{2+}, in the medium improves the heat resistance properties of the spores [103, 104]. This is assumed to be due to stabilisation of proteins by the DPA-calcium chelate. Manganese ions are also shown to increase the heat resistance [103, 105, 106]. The relative amounts of glucose, sucrose and calcium in the medium has been reported to be important. Addition of sucrose to a calcium-rich medium increased sporulation and the thermal resistance of *B.subtilis* spores [107]. Sodium chloride reduced the heat resistance of these spores. There have been contradictory reports on the effect of phosphate concentration on thermal resistance. High levels of phosphate have been reported to reduce the heat resistance of spores [108], but medium with 0.05% phosphate was shown to produce spores with higher heat resistance than unsupplemented medium [109].

Differing effects of storage of spore suspensions on heat resistance have been reported. The age of spore suspensions has been correlated with the degree of dehydration of the core. Newly formed and very old spores tend to show lower thermal resistance than average mature spores [110]. Methods used to clean spore crops effect their heat

resistance. Washing has the least effect, compared to sonication and enzyme treatments, but tends to lower resistance, probably due to removal of organic or inorganic protective materials. Excessive washing may interfere with spore coats [111].

1.5.2 Specific mechanisms of enhanced protein thermostability

In general, spores contain many of the enzymes present in vegetative cells. However, there are changes in the pattern of enzymes present, both prior to and during sporogenesis [7]. Seventy five to ninety percent of the soluble spore proteins of *B.subtilis* are synthesised during sporulation [112]. Studies on specific enzymes have attempted to account for differences between spore and vegetative cell enzymes [113]. Differences in antigenic or heat resistance properties are likely to be due to post-translational modification, the enzymes being coded for by the same genetic sequence [114].

Murrell [115] studied catalases of intact spores and spore extracts. In intact spores, the half life of the enzyme was 30 minutes at 100°C and it was resistant to 0.02M azide and 5 x 10^{-4} M cyanide. In the spore extracts, the catalase was no more stable than the vegetative cell enzyme. He concluded that the spore enzyme was identical to the vegetative cell enzyme, but was protected by the spore coat or internal spore structures. Disruption of spores in aqueous environments results in the dilution of ionic and organic compounds such as DPA, calcium and manganese which have a role in stabilisation of proteins *in vivo*. DPA is thought to exert its stabilisation effect by cross-linking with calcium, other metal ions and proteins [113]. Ionic effects were noted when purified glucose dehydrogenase (GDH) underwent a dimer to monomer interconversion between pH 6.5 and 8.0, leading to a 260-fold increase in heat resistance. Further enhancement of heat resistance was effected by the addition of high concentrations of NaCl to the medium. It was possible to stabilise the enzyme to the extent seen *in vivo* [116].

Although levels of NaCl needed for this effect are not present in the spore, an ionic or charge equivalent is possible. The heat resistant spore enzyme could be converted to the heat sensitive dimer by dialysis against a suitable buffer. Similarly, differences in the properties of spore and vegetative cell purine nucleotide phosphorylase can be accounted for by interconversions of different forms of the same protein species. Spore and vegetative forms of the aldolase enzyme of *B.cereus* have been studied. They appear to have similar kinetic and immunological properties and identical thermal stabilities in the absence of calcium. However, when calcium is added, the stability of the spore enzyme increases whereas the vegetative cell enzyme becomes more labile [116]. The transition of cell to spore enzyme often involves selected proteolysis, such as aldolases and purine nucleotide phosphorylases. The lower molecular weight derivatives of the cellular enzymes usually adopt the more stable conformation, possibly due to the shorter, and therefore stronger intramolecular bonds [116].

Amaki *et al* [117] investigated the thermostability and activity of the esterase enzyme of *B.stearothermophilus*. They replaced two free cysteine residues within the enzyme with serine or alanine by site-directed mutagenesis, producing 4 single mutations and 2 double mutations. All the mutants retained catalytic activity, demonstrating that neither of the cysteine residues were important in the active site of the enzyme. They found that replacement of both the cysteine residues led to an increase in the stability of the enzyme at 70°C. Free cysteine residues are known to be reactive, and the disulphide bridges are thought to be disrupted and lead to instability at high temperatures. In thermostable bovine Cu, Zn superoxide dismutase, replacement of the cysteine residue improves the enzyme stability at 70°C [118]. These observations suggest an important role for thiols in irreversible denaturation of proteins. It is possible that replacement of other unstable amino acids such as methionine, asparagine and glutamine residues may improve thermostability of other enzymes [119]. Replacement or blocking of such free reactive

amino acid residues is a possible mechanism of increased resistance of spore proteins compared to the corresponding cellular forms.

Suzuki [120] proposed a general principle of increasing thermostability. It has been demonstrated that even a single amino acid substitution in a protein can have a small effect on the thermostability of the protein; multiple substitutions have a cumulative effect. A strong correlation was found between the increased number of proline residues and the rise in thermostability of five *Bacillus* oligo-1,6-glucosidases [121]. A proline residue in a polypeptide chain restricts the flexibility of the backbone, both at the proline residue itself, and at the precedung residue. An increasing frequency of proline residues at β-turns led to restricted backbone conformation, and is a suggested mechanism for increasing protein thermostability. The proline residues are thought to cause the polypeptide chain to fold reversely and form hydrogen bonds with polar side chains of other amino acids. Proline residues were found to occur with increasing frequency in parallel with increasing thermostability of three oligo-1,6-glucosidases of *B.cereus*, *B.coagulans* and *B.thermoglucosidasius* [121]. Whilst proline residues were found to increase in an approximately linear fashion with an increase in thermostability, arginine also increased, but non-linearly. Contents of leucine and alanine roughly increased, while lysine, methionine, serine, isoleucine, aspartic and glutamic residues decreased with increasing thermostability. The extent of hydrophobic interactions increased, and the proportion of residues capable of hydrogen bonding reduced with increasing heat resistance. Ionic interactions were reduced as a result of improved thermostability [122, 123].

1.6 Bacterial α-glucosidases

Alpha-glucosidases catalyse the hydrolysis of terminal, non-reducing α-glycosidic bonds in short chain saccharides, produced by the action of other enzymes, such as amylases, on starch, releasing D-glucose. The substrate specificity of α-glucosidases varies depending on the source of the enzyme [124]. One group of enzymes has been termed maltases, since they have little, if any, activity towards PNPG, and high activity towards maltose [125]. The other group of α-glucosidases is less common, and includes enzymes that hydrolyse PNPG more rapidly than maltose [126, 127]. The α-glucosidase of *B.stearothermophilus* ATCC 12016 [128] preferentially hydrolyses maltose and maltotriose. It has no activity towards α-1,6 linkages, *ie.* isomaltose and isomaltosaccharides. Kelly *et al* [125] resolved two separate enzymes, a maltase and an α-glucosidase, in *B.licheniformis* NCIB 8549, differing in molecular weight and substrate specificity. They suggested that enzymes that have been reported to have broad substrate specificity have, in fact, comprised two or more distinct enzyme species. Generally, these enzymes degrade disaccharides and oligosaccharides quickly, and polysaccharides slowly, if at all. Alpha-glucosidases are found widely in animals, plants, and microorganisms, and are usually found in association with amylases, to effect the complete degradation of starch and other macromolecules [124]. Most bacterial α-glucosidases are intracellular, but some strains have extracellular and cell-bound forms. Most α-glucosidases have pH optima at approximately pH 7, or in the acidic region, although some are stable in the alkaline region. Molecular weights of α -glucosidases vary between 17 000 and 160 000. Alpha-glucosidases are generally inhibited by certain carbohydrates, such as glucose (end-product inhibition) and by heavy metal ions [124].

Nakao *et al* [129] reported isolation of a novel thermostable α-glucosidase from a thermophilic *Bacillus* from a hot spring. The enzyme had broad substrate specificity,

being capable of hydrolysing α-1,1, α-1,3, α-1,4 and α-1,6 glycosidic linkages. Differences in substrate specificity between this enzyme and other oligo-1,6-glucosidases could be due to specific amino acid substitutions near substrate binding and catalytic sites [129]. Nakao *et al* [130] cloned the α-glucosidase gene from thermophilic *Bacillus* sp. SAM 1606 and overexpressed it in *E.coli* transformants. This α-glucosidase had a molecular weight of 68 886 Da. Sequence similarity of this α-glucosidase was very high (62-65%) with oligo-1,6-glucosidases of *B.thermoglucosidasius* KP 1006 and *B.cereus* ATCC 7064 [122, 123]. The predicted folding of these 3 enzymes was highly conserved. Maltase from *Saccharomyces carlsbergensis* and α-amylase C from *Dictyoglomus thermophilum* showed 37% and 28% sequence identity respectively with the α-glucosidase of *Bacillus* sp. SAM 1606. The α-glucosidase of *Bacillus* sp. SAM 1606 showed no sequence similarity with mammalian and fungal α-glucosidases, suggesting the existence of at least 2 major classes of α-glucosidases.

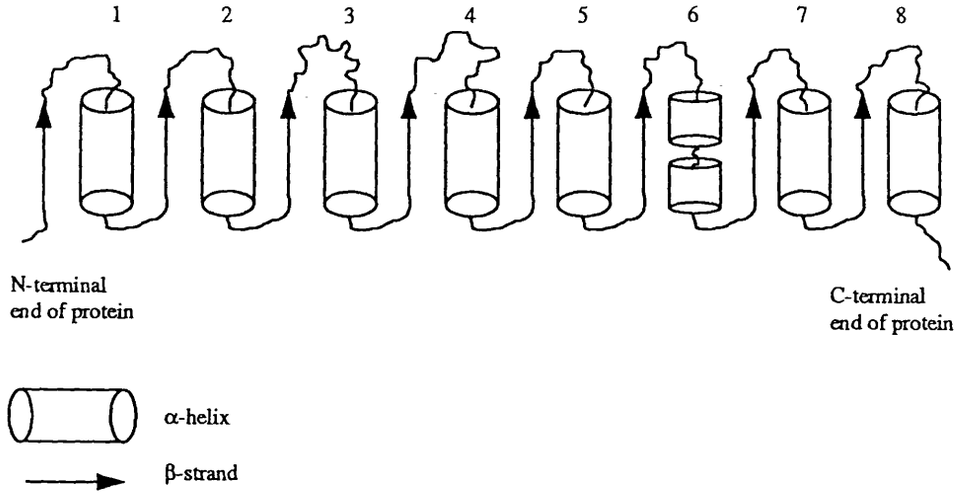
Suzuki *et al* [128, 131, 132, 133, 134, 135] studied α-glucosidases from a number of isolates from soil samples, including a new thermophilic *Bacillus* species, KP1006. These isolates were found to be very similar to *B.stearothermophilus*, but having a narrower pH and temperature range capable of supporting growth, slightly different physical appearance, and a greater ability to produce extracellular alpha-glucosidase. The α-glucosidase was produced in the cytoplasm during logarithmic growth. The level of enzyme decreased suddenly once the cells reached stationary phase. Alpha-glucosidase appeared in the culture broth in mid-logarithmic phase, and accumulated steadily throughout cultivation. Suzuki *et al* carried out further investigations into the characterisation of the purified enzyme [135]. They studied the effects of temperature and pH on the activity and stability of the enzyme. There was a sharp peak in it's activity at 75 °C, rapidly decreasing above and below this temperature. The pH for optimum activity was found to be between pH 5.0 and 6.0. The enzyme was stable over a wide pH range at 30°C, but had a much

narrower pH range for stability at higher temperatures. Alpha-glucosidase was inhibited by heavy metal irons, tris and histine, and was subject to end-product inhibition by glucose and PNP. Urlaub and Wober [126] studied the alpha-glucosidase enzyme of the mesophile, *B.amyloliquefaciens*. The enzyme was found to be an extracellular enzyme and was also bound to the cell membrane. The α-glucosidase of *B.circulans* was found in association with amylase, and the differing sensitivities of the two enzymes to glucose repression, (amylase being more sensitive than α-glucosidase), allowed the production of the amylase enzyme to be finely controlled [127].

Gottschalk [136] stated that α-glucosidases were sensitive to the conformation at carbon atoms 1, 2, 3 and 4 of their substrates because any change would either result in steric hindrance by replacing a hydrogen ion with a larger hydroxyl group, or would remove the hydroxyl group from its correct spatial positioning. Kelly and Fogarty [124] have reviewed the substrate specificity of a large number of microbial α-glucosidases. In an analysis of twelve bacterial α-glucosidases, ten had highest activity towards maltose, and of these, three had activity for the α-1,6 linkage. Four of these enzymes had aryl-α-D-glucoside activity, and none had significant alkyl-α-D-glucoside activity. The remaining two enzymes studied, from *B.amyloliquefaciens* and *Bacillus* sp. 7196, showed maximum activity towards sucrose and *p*-nitrophenyl-α-D-glucoside, respectively.

MacGregor and Svensson [137] studied the amino acid sequences and predicted protein secondary structures of the N-terminal domains of cyclodextrin glucanotransferases (CGTases), amylases and a yeast α-glucosidase (*Saccharomyces cerevisiae*). It is believed that the α-glucosidase and the CGTases may share the same super-secondary structure as the amylases, ie. an $(\alpha / \beta)_8$-barrel, which contains the active site. This structure consists of a barrel of 8 parallel β-strands surrounded by 8 α-helices (Figure 1.7) [138].

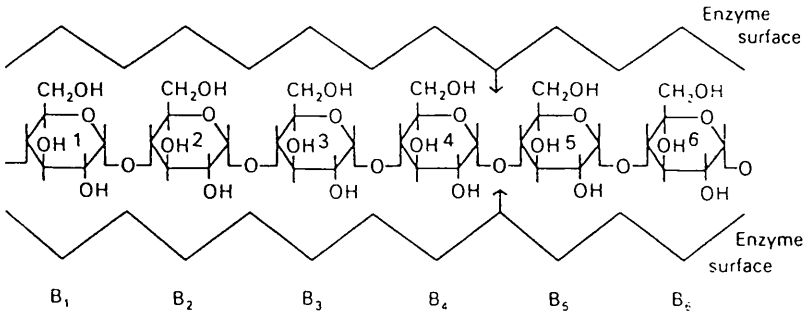
Figure 1.7. Schematic representation of structural features of the $(\alpha/\beta)_8$-barrel domain, common to α-amylase, CGTases and α-glucosidase. Loops 1 to 8 link the C-terminal strands to the N-terminal ends of adjacent helices, and carry amino acid residues that form the subsites of the enzyme active site [139].



The active site comprises a separate domain between a β-strand and an α-helix of the barrel. Alpha-amylases, CGTases and α-glucosidases all catalyse the cleavage of α-1,4-glycosidic bonds in malto-oligosaccharides or polysaccharides such as amylose and amylopectin. Local amino acid sequences near the active sites of the enzymes have been found to have similarities [140]. The tertiary structures of the $(\alpha / \beta)_8$-barrels of the α-glucosidase, CGTases and amylases are basically very similar [137]. Alpha-glucosidase contained conserved residues known to be important in Ca^{2+}-binding in α-amylases [138]. It has been suggested that α-glucosidases may contain Ca^{2+} ions. However, fully functional enzyme has been prepared in the presence of EDTA. Therefore it is unlikely that Ca^{2+} is required for enzyme activity [137].

Amino acids at the active sites of the enzymes are either important in catalysis of cleavage of the α-glycosidic bond, or binding of glucose residues of the substrate. Variations in specificity among α-amylases, CGTases and α-glucosidases, which all hydrolyse α-1,4 bonds, are due to differences in substrate-binding amino acid residues. Conserved amino acid sequences should predominate in regions responsible for binding substrates adjacent to the bond to be cleaved. The α-glucosidase requires an α-D-glucopyranose ring at the non-reducing end of the substrate, or it can accomodate other residues, such as the *p*-nitrophenyl group. This group presumably binds at subsite B_4 in the model of the active site (Figure 1.8), and if it is an α-D-glucopyranosyl group, will be subsequently released as α-D-glucose. There is much greater conservation of subsite B_4 between α-amylases and α-glucosidases.

Figure 1.8. Schematic representation of substrate binding at the active site of α-amylase, CGTase and α-glucosidase. B_1 to B_6 are subsites, each interacting with one glucose residue of the substrate. Arrows indicate catalytic amino acid residues between subsites B_4 and B_5. The glycosidic bond between rings 4 and 5 of the substrate would be cleaved [137].



The presence of a hydrophobic amino acid residue in the B_5 subsite of α-glucosidase may promote binding of the p-nitrophenyl group. Subsite B_2 may not be present in α-glucosidases that only catalyse hydrolysis of short chain oligosaccharides. The wide variation in action of amylases, CGTases and α-glucosidases is likely to be accounted for by differences in loop structures between the β-strands and α-helices, although key functional residues are conserved, and they share the general $(\alpha / \beta)_8$-barrel super-secondary structure [137, 138, 139].

In the search for an enzymatic indicator of spore viability, a wide range of extracellular and surface-associated enzymes of *B.stearothermophilus*, ATCC 7953, were evaluated. Inactivation of the α-glucosidase enzyme had superior correlation with spore kill than the other enzymes investigated. In addition, the substrate, 4-MUG, was stable following sterilising conditions, allowing its inclusion in the recovery medium of the BI.

More research is required to clarify the characterisation of α-glucosidases in terms of substrate specificity and other properties. Once the genetic sequence of these enzymes has been elucidated, this will aid further study of the three-dimensional structures and catalytic sites of alpha-glucosidases, and also enhance knowledge of potential mechanisms of thermostability in α-glucosidases from thermophilic organisms.

Chapter 2

GENERAL MATERIALS AND METHODS

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2.1 Apparatus

2.1.1 Glassware

Test tubes were rimless, 150mm x 16mm, of Pyrex glass, with aluminium caps (Oxoid, Basingstoke, England). Medical flat bottles of 500ml and 150ml volumes with polypropylene caps (FSA, Loughborough, England) were used. All volumetric glassware was of Grade B standard (FSA).

2.1.2 Disposable Plasticware

Sterile 9cm polystyrene petri-dishes (Bibby Sterilin Ltd., Stoke, England) were used for the growth and sporulation of bacteria on solid media. Sterile polystyrene 30ml centrifuge tubes (Bibby Sterilin Ltd.) were used for centrifugation and resuspension of bacterial cultures and enzyme suspensions.

2.1.3 Microscope

A phase contrast microscope (Wild, Switzerland) with x10, x40 and x100 (oil) objectives (Wild, Switzerland) was used in this work.

2.1.4 Balances

Weights below 2g were measured with an Oertling series 040 four digit balance. Weights above 2g were measured using an Oertling TP31 top pan balance.

2.1.5 Pipettes

A range of Gilson semi-automatic pipettes were used for measurements of small volumes of liquids.

Gilson P5000	for volumes between 1ml and 5ml.
Gilson P1000	for volumes between 0.2ml and 1ml.
Gilson P200	for volumes between 0.02ml and 0.2ml
Gilson P20	for volumes less than 0.02ml

Pipette tips (Anachem Ltd.) were recycled after use. This consisted of decontamination, washing and rinsing several times in distilled water, then packing into autoclave bags (DRG Ltd., Bristol), sealing and autoclaving for 15 minutes at 121°C.

2.1.6 Water

All water used was freshly glass double distilled water. It was sterilised by autoclaving at 121°C for 15 minutes in 100ml volumes in 150ml glass medical flat bottles where required to be sterile.

2.1.7 Chemicals and reagents

All chemicals used in this study were of analytical reagent grade and supplied by Sigma Chemical Co., Poole, England, unless stated otherwise.

2.2 Preparation of Media for General Use

2.2.1 Nutrient Agar (NA)

Nutrient Agar (Lab M, Amersham, Bury, England) was used as a growth medium for the determination of viable counts of spore suspensions. It was used in preference to tryptone soy agar (TSA) which tends to cause rapid and diffuse growth of *Bacillus stearothermophilus* and render it difficult to count discreet colonies.

Formula

Peptone	5.0g/l
Beef extract	3.0g/l
Sodium chloride	8.0g/l
Agar No.2	12.0g/l

Final pH 7.3 ± 0.2 at 25°C.

To prepare NA, 28g of powder was weighed out and dispersed in 1 litre of distilled water. It was allowed to soak for 10 minutes, swirled to mix, then autoclaved for 15 minutes at 121°C. It was cooled to handling temperature (50-60°C), then mixed and dispensed in approximately 20ml volumes into sterile 9cm polystyrene petri-dishes. The agar was allowed to cool, then stored inverted in the dark at 4°C for up to 1 month. The agar plates were overdried inverted at 37°C for 1 hour prior to use.

2.2.2 *Basic growth medium (BM)* [133]

This medium was used for growth of vegetative cell cultures in experiments in which the effect of carbohydrates and other nutrients on the growth and production of alpha-glucosidase was to be compared.

Peptone (Oxoid)	25g
Meat extract (Lab M, Amersham)	2g
Yeast extract (Difco Laboratories, Detroit, USA)	3g
K_2HPO_4	3g
KH_2PO_4	1g
Distilled water	to 1 litre

The pH of the medium was adjusted to pH 7.3 ± 0.1 and then sterilised by autoclaving for 15 minutes at 121°C.

2.2.3 *Chemically-defined medium (CDM)*

This medium was used in experiments in which the concentration of carbohydrates in the medium were to be controlled, to assess effect of such compounds on the alpha-glucosidase activity. Table 2.1 shows the composition of chemically defined medium [141].

Stock solutions of the components of CDM were prepared as shown in Table 2.3. The solutions were filter sterilised using a Swinnex filtration unit (Millipore Corporation, Bedford, Massachusetts) and a 0.2µl filter (Nucleopore Corporation Filtration Products,

Pleasanton, California). The solutions were stored in sterile glass medical flat bottles prior to use.

Table 2.1. Composition of CDM

Constituent	*Molarity*
L-Glutamic acid	2.4×10^{-3}
D-Glucose	1.0×10^{-3}
L-Methionine	4.5×10^{-5}
$MgCl_2.6H_2O$	1.8×10^{-5}
$MnCl_2.4H_2O$	1.5×10^{-5}
$CaCl_2$	5.5×10^{-5}
$FeSO_4.7H_2O$	1.0×10^{-5}
Nicotinic acid (sodium salt)	6.0×10^{-7}
Thiamine hydrochloride	2.5×10^{-8}
Biotin	8.0×10^{-10}
$Na_2HPO_4.2H_2O$	1.76×10^{-2}
KH_2PO_4	7.3×10^{-3}

The medium was prepared by mixing the required volumes of the stock solutions as stated in Table 2.2. For production of solid CDM, 500ml of the double strength solution was added to 500ml of 3% agar (sterilised for 15 minutes at 121°C) at about 50°C, and mixed thoroughly. The medium was then dispensed in approximately 20ml volumes into 9cm disposable petri dishes. For production of liquid medium, 500ml of double strength solution was added to 500ml of sterile distilled water and mixed thoroughly.

Table 2.2. Volumes of stock solutions required for the preparation of CDM

Component	*Volume required*
Solution I	100ml
Solution II	100ml
Solution III	0.5ml of each solution
Solution IV	1.5ml
Solution V	1.5ml
Solution VI	1.5ml
SDW	to 500ml
3% Agar (Oxoid)	500ml

Table 2.3. Composition of stock solutions for the preparation of CDM

Solution	*Component*	*Amount (g)*	*Water to (ml)*
I	L-Glutamic acid	0.450	
	D-Glucose	0.180	100
	L-Methionine	0.007	
II	KH_2PO_4	0.993	
	Na_2PO_4	3.131	100
III	$MgCl_2.6H_2O$	0.740	100
	$MnCl_2.4H_2O$	0.600	100
	$CaCl_2$	1.221	100
	$FeSO_4.7H_2O$	0.560	100
IV	Thiamine HCl soln.(0.8g/l)	1.0ml	100
V	L-Biotin soln.(0.2g/l)	1.0ml	100
VI	Nicotinic acid soln.(0.9g/l)	1.0ml	10

2.2.4 Tryptic Soy Broth (TSB)

This was used for growth of the stock vegetative culture and to produce cultures used for the inoculation of the sporulation media.

Formula

Tryptone (Pancreatic digest of casein)	17g/l
Soytone (Papaic digest of soybean meal)	3g/l
Dextrose	2.5g/l
Sodium chloride	5g/l
Dipotassium phosphate	2.5g/l

Final pH 7.3 ± 0.2 at 25°C.

To prepare TSB, 30g of powder (Difco) was dissolved in 1 litre of distilled water, and then dispensed into approximately 100ml volumes in 150ml medical flat bottles. The medium was sterilised by autoclaving for 15 minutes at 121°C.

2.3 Preparation of Buffer Solutions

2.3.1 50mM potassium phosphate/5mM EDTA buffer pH 7.4

The following solutions were prepared.

Solution A	0.05M K_2HPO_4 and 0.005M EDTA
Solution B	0.05M KH_2PO_4 and 0.005M EDTA

2304ml of solution A was mixed with 696ml of solution B. The pH was adjusted to pH 7.4 with 1M sodium hydroxide solution or 1M hydrochloric acid.

2.3.2 Sørensens phosphate buffers

A range of Sørensens buffers were prepared of pH values between 5.0 and 8.2, using varying volumes of 2 stock solutions (Solutions I and II).

Table 2.4. Preparation of Sørensen's phosphate buffers, pH 5.0-8.2 [141]

pH of buffer	*Volume of solution I*	*Volume of solution II*
5.0	99.8	0.2
5.2	98.0	2.0
5.4	96.7	3.3
5.6	94.8	5.2
5.8	91.9	8.1
6.0	87.7	12.3
6.2	81.5	18.5
6.4	73.2	26.8
6.6	62.7	37.3
6.8	50.8	49.2
7.0	39.2	60.8
7.2	28.5	71.5
7.4	19.6	80.4
7.6	13.2	86.8
7.8	8.6	91.4
8.0	5.5	94.5
8.2	3.3	96.7

Solution I 1/15M monopotassium phosphate (9.08g KH_2PO_4 per litre)

Solution II 1/15M disodium phosphate (11.88g $Na_2HPO_4 .2H_2O$ per litre)

2.4 Spore Production

2.4.1 Production of stock vegetative culture

B.stearothermophilus spore strip stock culture 213, June 1990, was used to inoculate 2 x 75ml TSB in 250ml conical flasks. A broth sterility test was carried out to check for the absence of contamination of the TSB, by incubating portions of the broth at 60°C, 37°C and 25°C to test for the growth of thermophilic bacteria, mesophilic bacteria or fungal contaminants, respectively. A negative result was obtained prior to inoculation with the spore strip.

The cultures were incubated at 60°C for 18 hours. They were examined under phase microscope to check that they comprised rod-shaped vegetative cells, and stained to ensure Gram-positive cells were present. The cultures were streaked onto TSA (Lab M, Amersham) plates to check for culture purity. Approximately 1ml volumes of stock vegetative culture were aseptically transferred to sterile plastic straws and stored in liquid nitrogen.

2.4.2 Production of spore suspension from stock vegetative culture

2.4.2.1 Pre-sporulation stage

One "straw" of frozen stock vegetative culture was thawed and used to inoculate 75ml TSB in a 250ml conical flask. This was incubated unshaken at 60°C for 12 hours.

2.4.2.2 Sporulation stage

Sporulation agar was prepared in 3 litre batches, 24 hours prior to inoculation, according to 3M Brookings Manufacturing Standard PMEO - 2886.

Formula

Yeast Extract (Difco)	12g
Nutrient Broth (Difco)	24g
Agar (Difco)	60g
Manganese Chloride	0.3g
Distilled Water	3 litres

The nutrient broth, yeast extract and the manganese chloride were weighed out and added to 3 litres of distilled water and stirred to dissolve. The medium was adjusted to pH 7.2 ± 0.1 with 10% sodium hydroxide solution.

Ten grams of the agar was weighed out and added to each of twelve 500ml glass flat bottles. 500ml of the medium was added to each bottle and shaken to suspend the agar. The sporulation agar was sterilised at 121°C for 30 minutes. It was cooled to handling temperature and dispensed in 20ml volumes into approximately 150 disposable petri dishes (Sterilin Products Ltd.) (9cm diameter). The sporulation plates were inoculated with 0.5ml of the 12 hour vegetative culture, and incubated at 60°C. Samples were examined under phase microscope at regular intervals to determine the extent of sporulation. Maximum sporulation was observed after 72 hours incubation.

2.4.2.3 Harvesting

Five millilitres of ice cold sterile distilled water was pipetted onto the sporulation plates. Spores were loosened from the agar with a sterile glass spreader, and the suspension was transferred to a sterile glass container, stored on ice.

2.4.2.4 Cleaning

Spore suspension was transferred to sterile centrifuge tubes, balanced using ice cold sterile distilled water and centrifuged at 4000 rpm at 4°C for 20 minutes.
Supernatants were discarded, and pellets resuspended in 10ml ice cold sterile water per centrifuge tube and combined. Centrifugation was repeated.
The pellet formed was in 2 or 3 layers [142] :

Bottom layer (dark) - cell debris and any intact vegetative cells containing spores.
Top layer (cream / brown) - intact, unsporulated vegetative cells.
Middle layer (light grey / pink) - free spores.

The top layer of the pellet was removed by gently swirling the supernatant to resuspend the loosely-packed top layer. The supernatant was discarded. 10ml of ice cold sterile water was added. Approximately 95% of the middle layer was resuspended. The suspension was decanted into a sterile centrifuge tube. Centrifugation was repeated and any layers were removed as described above. The pellet was resuspended in ice cold sterile distilled water (various volumes used). The spore suspension was stored at 4°C.

2.5 Spore population assays

Total count, viable count and heat-shocked count were performed on each of the spore crops produced.

2.5.1 Total count

A total count was performed using a microscope method (Neubauer Counting Chamber). A coverslip was placed on the counting chamber to produce Newton's Rings. An

appropriate dilution of the spore suspension was prepared to give an approximate count of 10 spores per small square of the chamber, and 1 drop was allowed to be drawn under the coverslip by capillary action. The slide was allowed to settle for 10 minutes, then observed under a x40 objective and a mean count of phase bright spores in 80 small squares of the counting chamber was taken.

Depth of counting chamber	=	0.1mm
Area of 1 small square	=	$1/400mm^2$
Volume of each small square	=	2.5×10^{-7}
Total Count per ml	=	mean count x Dilution factor / 2.5×10^{-7}

2.5.2 Viable count

Viable counts (*ie.* the number of viable organisms in a spore suspension) were performed using the spread plate method. Appropriate 10-fold serial dilutions (0.5ml + 4.5ml) of the spore suspension were carried out in sterile distilled water to give a final concentration of 200-750 spore per ml. Before each dilution, the suspension was mixed using a whirlimixer (Fisons Scientific Equipment, Loughborough, England). 0.2ml volumes of the final dilution (and other dilutions if necessary to ensure countable number of colonies on the plates) were pipetted onto 5 replicate NA plates and spread over the surface using a glass spreader. The plates were allowed to stand for 10 minutes to allow the liquid to be absorbed. The plates were then inverted and incubated at 60°C for 5 days. The number of colonies on each plate was recorded, and the mean count of the 5 replicates was used to calculate the number of spores per ml present in the original suspension.

2.5.3 Heat-shocked count

The heat-shocked count technique consisted of exposing suitable concentrations of the spore suspension to 100°C in a boiling water bath for 10 minutes, cooling in an ice water bath for 2 minutes then carrying out a viable count as described above.

2.5.4 Growth index (GI)

The growth index was calculated for each of the spore crops.

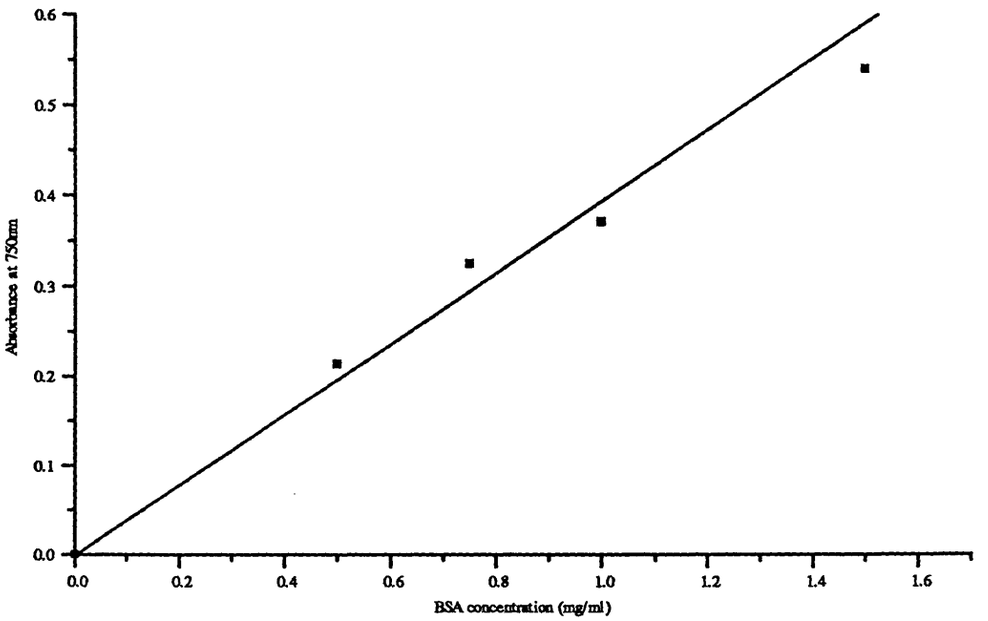
Growth index (GI) = Viable count / Total count x 100 (%)

2.6 Protein assay

A DC (Detergent Compatible) Protein Assay (modified Lowry assay [143]) was used (Bio-Rad, California). A standard curve was produced using concentrations of bovine serum albumin (BSA) between 0 - 1.5 mg/ml. 100µl of sample was pipetted into a test tube. Five hundred microlitres of Reagent A (alkaline copper tartrate solution) was added, and the mixture vortexed. Four millilitres of Reagent B (dilute folin reagent) was added and the mixture vortexed. The mixture was incubated for 15 minutes at room temperature. The absorbance was recorded at 750nm.

Figure 2.1. Calibration curve for protein assay.

The absorbance at 750nm of BSA (0-1.5 mg/ml) in phosphate-buffered saline pH 7.3.



The absorbance of the unknown samples were treated as above. The protein concentration was determined from the calibration plot (Figure 2.1).

Chapter 3

CHARACTERISATION OF THE α -GLUCOSIDASE ENZYME OF BACILLUS STEAROTHERMOPHILUS

Chapter 3

CHARACTERISATION OF THE α -GLUCOSIDASE ENZYME OF BACILLUS STEAROTHERMOPHILUS

3.1 Introduction

Although the 1291 Attest™ Rapid Readout Biological Indicator has been successfully marketed since 1990, there are few data available as to the fundamental properties of the spore α-glucosidase utilised in the product, and no attempt has been made to optimise it's activity. Some work has been previously carried out concerning the production of α-glucosidases by other thermophilic and mesophilic bacteria [126, 128, 131, 132, 133, 134, 135]. However, the localisation of α-glucosidase and its function within the spore have not previously been investigated. This chapter details the characterisation of the spore α-glucosidase and a comparison of its properties with those of the vegetative form of the enzyme, and with other characterised α-glucosidases, was made.

3.2 Materials and Methods

3.2.1 Para-nitrophenyl-α -D-glucoside (PNPG) assay

Alpha-glucosidase in spore and vegetative cell extracts was assayed using spectrophotometric detection of *p*-nitrophenol (PNP) formed from *p*-nitrophenyl-α-D-glucoside (PNPG) by the action of the enzyme. The coloured product that is measured in this assay, produced by cleavage of the attached glucose residue, is the nitrophenolate ion, which protonates to the colourless form in acidic conditions. Therefore for this assay system it is vital that the extinction coefficient that is applied at different pH values takes into account the pK_a of ionisation ($pK_a = 7.15$).
The change in absorbance of the reaction mixture was measured at 410nm. A scan of absorbance of the reaction mixture was carried out at wavelengths between 300 and

550nm to confirm that 410nm was the appropriate wavelength to use. The reaction mixture absorbs strongly at about 330nm, possibly due to the complex nature of the reaction mixture *ie.* contains enzyme and buffer salts, as well as the product of the reaction (PNP) which absorbs at the 400nm wavelength. The wavelength chosen for absorbance measurements was 410nm, at a slightly longer wavelength than λ_{max}, to reduce any possible interference from the other peak at 330nm.

A Pye-Unicam UV/VIS kinetics spectrophotometer was used for all absorbance measurements. Solutions of PNPG and enzyme extract were prepared according to the individual experiment. The PNPG, phosphate buffer, and the cuvettes (1ml matched quartz, Sciensco) were pre-heated to the reaction temperature. The reaction was started by the addition of the enzyme to the other reactants in the cuvette, mixing the contents by inverting the cuvette twice. The blank cuvette contained the PNPG and buffer solution only. Absorbance was recorded automatically at 15 second intervals at 410nm in a temperature-controlled sample chamber. The temperature of the chamber was monitored with a copper/constantan thermocouple (Fluke, J/K). The linear reaction velocity was calculated from the gradient of the initial linear portion of the graph, and used to determine the α-glucosidase activity (mol PNP/min/mg protein).

3.2.2 Methylumbelliferyl-α -D-glucoside (MUG) assay

Alpha-glucosidase in spore or vegetative cell suspensions or crude extracts was assayed by the fluorimetric detection of 4-methylumbelliferone (4-MU) released from the hydrolysis of 4-methylumbelliferyl-α-D-glucoside by α-glucosidase. A fluorescent product emits a constant fraction (quantum yield, Q_f) of the light energy it absorbs, but at a longer wavelength than the incident light. Unlike the use of the absorbance coefficient in absorbance measurements, there is no such standard that can relate fluorescence to units of concentration of a compound, even when experimental

conditions are manipulated to give a linear relationship between the two parameters. Therefore conversion of fluorescent values to concentration of a fluorescent compound must be achieved by use of known concentrations of the fluorescent product over the range expected in the assay.

Fluorimetric methods have the potential to be at least two orders of magnitude more sensitive than the corresponding absorbance assay. Methylumbelliferone (MU) can be reliably measured at 10^{-10}M concentration. However, a major disadvantage in the use of methylumbelliferone in enzyme assay systems is the fact that the fluorescent properties of MU are solely due to the anionic form of the compound, which is formed at high pH. Therefore this is not suitable for the detection of many hydrolytic compounds which are active in acidic conditions. This means that for many assays the enzyme-catalysed reaction is carried out at low or neutral pH, the reaction stopped and the fluorescent anion is subsequently formed by the addition of a strong alkaline buffer. This type of discontinuous assay system is not suitable for the determination of kinetic parameters, where the initial linear reaction velocity of the enzyme-catalysed reaction is to be determined and errors inherent in discontinuous assays are unacceptable. Therefore in this work, for determination of kinetic parameters, and the comparison of the linear reaction velocity of α-glucosidase under different conditions of temperature and pH, the PNPG assay was used. For the remaining analysis of α-glucosidase, the fluorimetric assay was used. A continuous method was employed, where the fluorescence produced was measured at the experimental pH (pH 7.4). Although this method reduced the potential sensitivity of the fluorescence assay, due to low inherent fluorescence at pH 7.4, it was possible to use this assay for screening a large number of spore suspensions and cell cultures directly for extracellular α-glucosidase activity, which was not possible with the PNPG assay due to high absorbance of the culture media at the wavelength employed for the assay (410nm). The buffer or culture medium (depending on the experiment) was pipetted into the wells of a 96-well microtitre plate (Costar, Cambridge, Massachusetts). The

appropriate volume (50µl, unless stated otherwise in individual experiment) of 4-MUG solution (0.1 g/l) was added. The spore or vegetative cell suspension or crude extract was added, and the reaction mixture was mixed by drawing it up and down several times in the pipette tip. The microtitre plate was incubated for 30 minutes at 60°C. The fluorescence (λ_{ex} 360nm, λ_{em} 455nm)of each sample was measured immediately using a plate-reading fluorimeter (Fluoroskan II, Titertek).

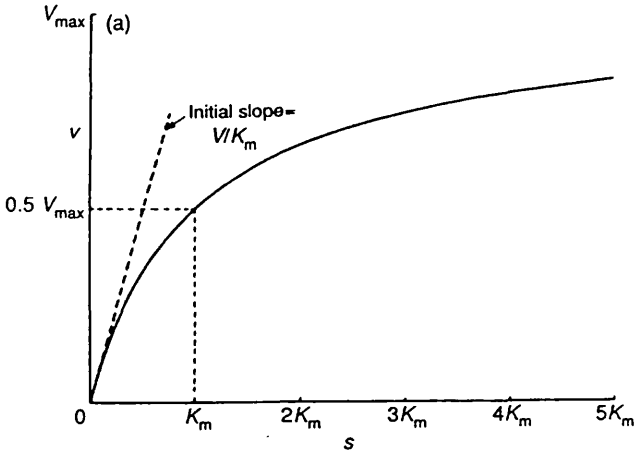
3.2.3 Treatment of results

For most enzyme-catalysed reactions, the rate of reaction, measured by formation of product, or depletion of substrate against time, can be represented as in Figure 3.1 [145]. Following an initial period of linearity, that is used to calculate the linear reaction velocity, the product formation decreases due to several possible factors, including substrate depletion or end-product inhibition. For Michaelis-Menten theory to be applied, the initial reaction velocity must be directly proportional to the enzyme concentration. Departure from the hyperbolic relationship predicted between initial reaction velocity and substrate concentration cen be caused by high substrate inhibition or apparent negative co-operativity, in which binding of one substrate molecule leads to a reduction in affinity of the enzyme for subsequent substrate molecules [145].

The Michaelis-Menten equation relates reaction velocity, v, to substrate concentration, s (*Equation 3.1*).

$$v = V_{max}\, s \,/\, K_m + s \qquad \textit{Equation 3.1}$$

Figure 3.1. Plot of initial velocity, *v*, against substrate concentration, *s*, for a reaction obeying the Michaelis-Menten equation [145]. K_m is the substrate concentration required to achieve half the maximum velocity of the enzyme-catalysed reaction, assuming that all other reactants are saturating. V_{max} is the maximum velocity of the enzyme-catalysed reaction when all reactants are saturating.



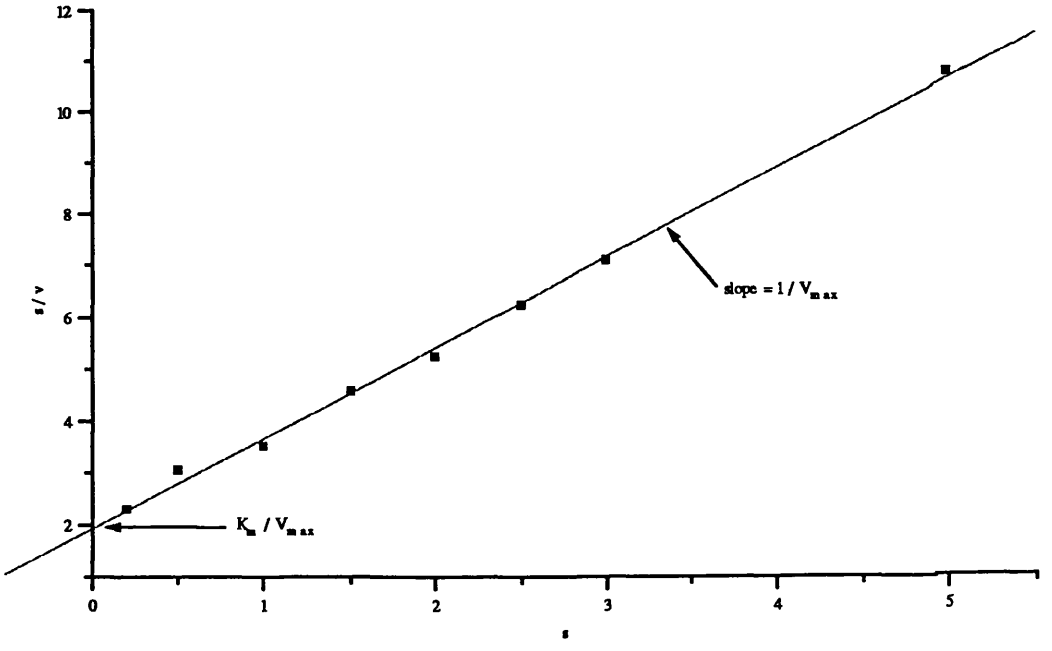
The Michaelis-Menten equation can be re-arranged into 3 different linear expressions that can be used to plot straight line graphs, from which values of K_m and V_{max} can be estimated [145]. These graphical methods, the Lineweaver-Burk plot ($1/v$ against $1/s$), the Eadie-Hoftsee plot (v against v/s) and the Hanes plot (s/v against s), have been proven to be inaccurate in determining K_m and V_{max} values, and should only be used as methods for displaying results [146].

3.2.3.1 Determination of kinetic parameters using the Direct Linear Plot method [147, 145]

The kinetic parameters, K_m and V_{max}, were determined using the direct linear plot, both constructed manually and by computer. The α-glucosidase activity was

determined by the PNPG assay (*3.2.1*) using a series of substrate concentrations; 0.2mM, 0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM and 5.0mM. The linear reaction velocity was calculated from the gradient of the initial portion of the reaction profile for each substrate concentration. A Hanes plot (*s/v* against *s*) was initially constructed to visually inspect the linearity of the data obtained (Figure 3.2).

Figure 3.2. Hanes plot for determination of kinetic parameters, K_m and V_{max}. Reaction was carried out with crude vegetative cell extract in 5mM PNPG in phosphate/EDTA buffer pH 7.4 at 60°C. Data used to construct plot are shown in Table 3.1.



3.2.3.1a Direct Linear Plot - manual method [145, 147]

The negative value of *s* was plotted on the horizontal axis. The corresponding value of *v* was plotted on the vertical axis. The two points were joined with a straight line, and the line was continued into the first quadrant. This was repeated for all the pairs of (*v*, *s*) values. With an ideal set of data, these lines would intersect at one point. However, due to experimental error, there were a number of intersections, related to the number of (*v*, *s*) values used.

Number of intersections = $n(n-1)/2$

n = number of pairs of (*v*, *s*) values (number of lines)

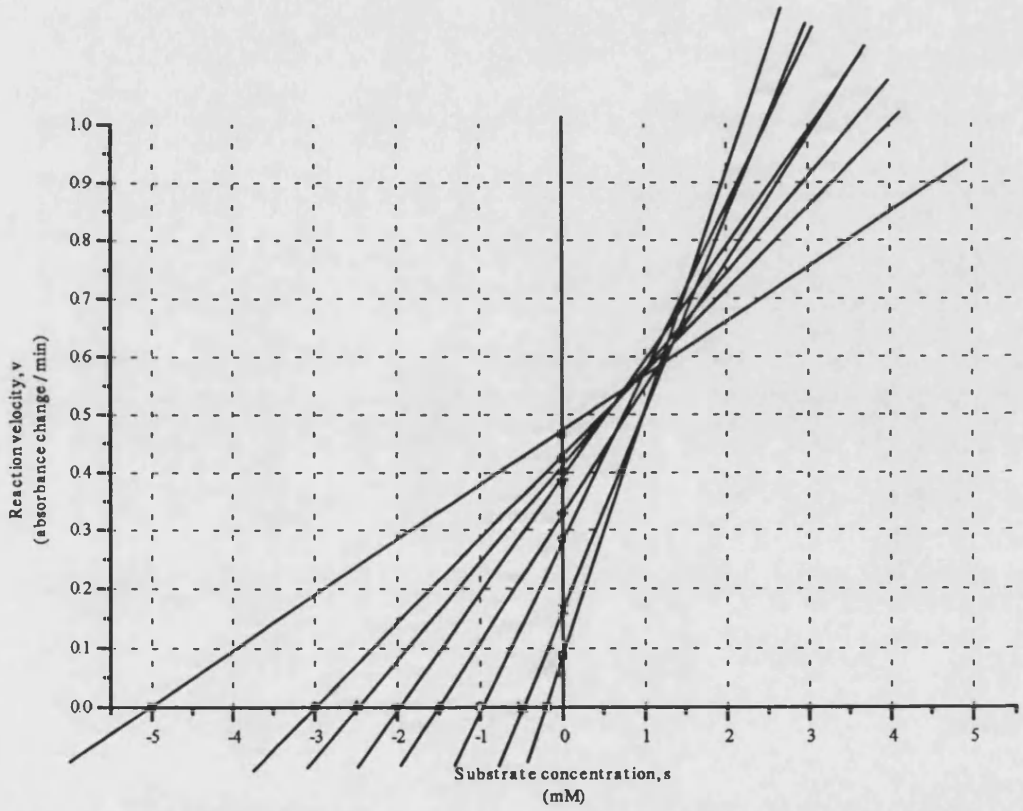
Table 3.1. Substrate concentrations and linear reaction velocities used in the construction of the direct linear plot. Reaction was carried out with crude vegetative cell extract (3.75×10^{-4} mg protein) in 5mM PNPG in phosphate/EDTA buffer pH 7.4 at 60°C.

Substrate concentration, s (mM)	*Velocity, v (absorbance change/min at 410nm)*
5.0	0.465
3.0	0.424
2.5	0.401
2.0	0.382
1.5	0.328
1.0	0.284
0.5	0.164
0.2	0.087

The median intersection point (points were ranked from left to right to obtain K_m and from bottom to top to obtain V_{max}) had the co-ordinates of the best-fit value of K_m

and V_{max}. The following example of a direct linear plot (Figure 3.3) was constructed using the substrate concentrations and linear reaction velocities shown in Table 3.1.

Figure 3.3. Direct Linear Plot to determine K_m and K_{max} values of α-glucosidase catalysed reaction - linear reaction velocity against substrate concentration. Reaction was carried out with crude vegetative cell extract in 5mM PNPG in phosphate/EDTA buffer pH 7.4 at 60°C.



3.2.3.1b Direct Linear Plot - computer method

Pairs of (v, s) values were generated in the usual manner. The data were analysed using "Enzpack 3" - version 3.0 (Peter A. Williams and Bogus N. Zaba. School of Biological Sciences. University College of North Wales. Bangor. UK. Published and distributed by BIOSOFT. 22 Hills Road. Cambridge. UK. © 1989. P.A.

Williams. EGA Graphics.). This allowed the calculation of kinetic parameters by the Direct Linear Plot method, and also the Hanes plot, Lineweaver-Burk plot, and the Eadie-Hoftsee plot.

3.2.4 SDS-PAGE system for molecular weight determination

Analysis of proteins is carried out in polyacrylamide gels in combination with heat and sodium dodecyl sulphate (SDS) to dissociate proteins into their individual polypeptide subunits. The amount of SDS bound is almost always proportional to the molecular weight of the polypeptide. The SDS-polypeptide complexes formed migrate through the polyacrylamide gels according to the size of the polypeptide. The use of markers of known molecular weights allows estimation of the molecular weight of the polypeptides [148].

An SDS-PAGE minigel system (Pharmacia-Biotech, Uppsala, Sweden) was used to separate the proteins in the spore and vegetative cell extracts, and to determine their molecular weights. The following components of the SDS-PAGE system were prepared.

3.2.4.1 Preparation of gels and buffer solutions

Separating gel

Deionised water	1.3ml
1.5M Tris, pH 8.8	3.75ml
10% sodium dodecyl sulphate (SDS)	300µl
Acrylamide stock solution (30% w/v acrylamide & 0.8% w/v bisacrylamide)	4.8ml
Tetramethylethylenediamine (TEMED)	28µl
Ammonium persulphate (APS)	40µl

Stacking gel

Deionised water	6.4ml
0.5M Tris, pH 6.8	3.0ml
10% SDS	120µl
Acrylamide stock solution	2.0ml
TEMED	32µl
APS	40µl

Running buffer

A stock solution of 10 x strength of the running buffer was prepared as below, and diluted 1 in 10 in DW prior to use:

SDS	10g
Tris	30g
Glycine	143g
Deionised water	to 1 litre

Sample buffer

0.5M Tris, pH 6.8	2.4ml
10% SDS	4.0ml
Glycerol	2.0ml
0.5% bromophenol blue	1.0ml

3.2.4.2 Sample and molecular weight marker preparation

Sample	36µl
Sample buffer	40µl
β-mercaptoethanol	4µl

Low range molecular weight markers	5µl
Sample buffer	20µl
Distilled water	15µl

The sample and marker mixtures were incubated at 60°C for 5 minutes, before 20µl of each mixture was loaded into each lane of the gel. The composition of the low range molecular weight markers (Bio-Rad, California) is shown in Table 3.2.

Table 3.2. Molecular weights of proteins in Biorad low range molecular weight marker

Protein	*Molecular weight*
Phosphorylase B	106 000
Bovine serum albumin (BSA)	80 000
Ovalbumin	49 500
Carbonic anhydrase	32 500
Soybean trypsin inhibitor	27 500
Lysozyme	18 500

3.2.4.3 Gel staining

Stain - Coomassie Brilliant Blue R-250

Coomassie blue	0.5g
Methanol	200ml
Acetic acid	50ml
Deionised water	250ml

Destaining solution

Methanol	10%
Acetic acid	25%
Deionised water	to 100%

The gel was stained overnight in the Coomassie blue stain and destained for approximately 1-2 hours, prior to being dried onto filter paper, or being scanned.

3.2.5 Gel filtration for molecular weight determination

Molecular weight determination by gel filtration is based on the ability of gel filtration media to separate molecules according to size. The elution volume of samples from a gel filtration column were used to calculate the corresponding K_{av} value (elution volume parameter), which was compared to the K_{av} value of a number of molecular weight standard proteins.

Sephadex G-200 gel filtration medium (Pharmacia-Biotech, Uppsala, Sweden) was allowed to swell in three times the expected gel bed volume of running buffer (Sorensen's phosphate buffer pH 7.4) for 5 days at room temperature prior to packing of the column. 1g dry weight of Sephadex G-200 was equivalent to 30ml gel bed volume. A C26/40 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and AC26 adaptor were used.

3.2.5.1 Calibration of Sephadex G-200 gel filtration column

The solutions of molecular weight markers (Pharmacia-Biotech, Uppsala, Sweden) were freshly prepared in phosphate buffer pH 7.4. The flow rate was calculated in terms of weight of buffer collected from the column per minute. Five hundred microlitres injection volume was used for each sample. Blue dextran was injected, and the column void volume (column void volume = elution volume for blue dextran) was calculated from the chart recording (UV absorbance at 280nm). The marker proteins were injected in two groups to allow sufficient resolution. Run 1 consisted of catalase, albumin and chymotrypsinogen A; run 2 consisted of ferritin, ovalbumin and ribonuclease A. The elution volume for each protein was measured from the start of the sample application to the column to the centre of the peak, measured by the intersection of the two tangents drawn to the sides of the peak. The K_{av} value was calculated for each peak (*Equation 3.2*).

$$K_{av} = V_e - V_o / V_t - V_o \qquad \textit{Equation 3.2}$$

V_e = elution volume for protein

V_o = column void volume = elution volume for blue dextran

V_t = total bed volume

The K_{av} value was plotted against the corresponding molecular weight for each protein (on a logarithmic scale). The best fit straight line was determined. The molecular weight of the applied samples was calculated from their K_{av} values.

3.2.6 Western blotting

Electrophoretically separated proteins are transferred from a gel to a solid support (usually a nitrocellulose filter) and probed with specific reagents. The specific reagents are usually antibodies that react with epitopes of the required protein attached to the solid support [148].

Proteins were immunolabelled with the anti-spore α-glucosidase monoclonal antibody, to assess the cross-reactivity of the spore and vegetative cell extracts.

The following buffer solution was prepared and chilled thoroughly prior to use.

Transfer buffer

Tris	3g
Glycine	14.4g
Methanol	200ml
Distilled water	to 1 litre

The samples of spore and vegetative cell extract were run on an SDS-PAGE gel, with molecular weight markers (*3.2.3*). Following electrophoresis, the gel was rinsed in

transfer buffer for 1 hour to equilibrate and to remove reactive buffer salts from the gel which may hinder electrophoretic transfer of the proteins. The Bio-Ice cooling unit was filled with distilled water and frozen before use. One piece of nitrocellulose membrane (Bio-Rad, California) and two pieces of Whatman 3M filter paper (Whatman Ltd., Maidstone, England) were cut to the same dimensions as the gel. The orientation of the gel on the membrane was carefully marked with a soft pencil. The membrane, filter paper and fibre pads were saturated with cold transfer buffer, prior to being assembled in the gel holder according to manufacturer's instructions. The Bio-Ice unit and the gel holder were placed in the buffer chamber, which was filled with cold transfer buffer. The transfer was carried out at 100V for 1 hour following manufacturer's instructions. The nitrocellulose membrane was either used immediately or was stored at -18°C.

3.2.7 Immunolabeling of proteins on nitrocellulose membrane

The nitrocellulose membrane was incubated in blocking buffer for 1 hour at room temperature.

Blocking buffer

Tween 20	0.1%
BSA	0.1%
Tris-buffered saline	to 100%

Tris-buffered saline (TBS)

Sodium chloride	0.9%
Tris	0.12%
Distilled water	to 100%

The blocking buffer was removed, and the membrane was incubated in a 1 in 50 dilution of the mouse anti-spore α-glucosidase monoclonal antibody in the blocking

buffer for 4 hours at room temperature. The primary antibody solution was removed, and the membrane was rinsed by addition of fresh portions of blocking buffer and agitating for 30 minutes. A 1 in 50 dilution of the secondary goat antimouse gold-conjugated antibody (British Biocell International, Cardiff) was added and incubated at room temperature for 2 hours. The membrane was rinsed with TBS. A red colouration of bands that had been labelled with the antibody was observed.

3.2.8 Chromatographic analysis of hydrolysis of various carbohydrates by α - glucosidase

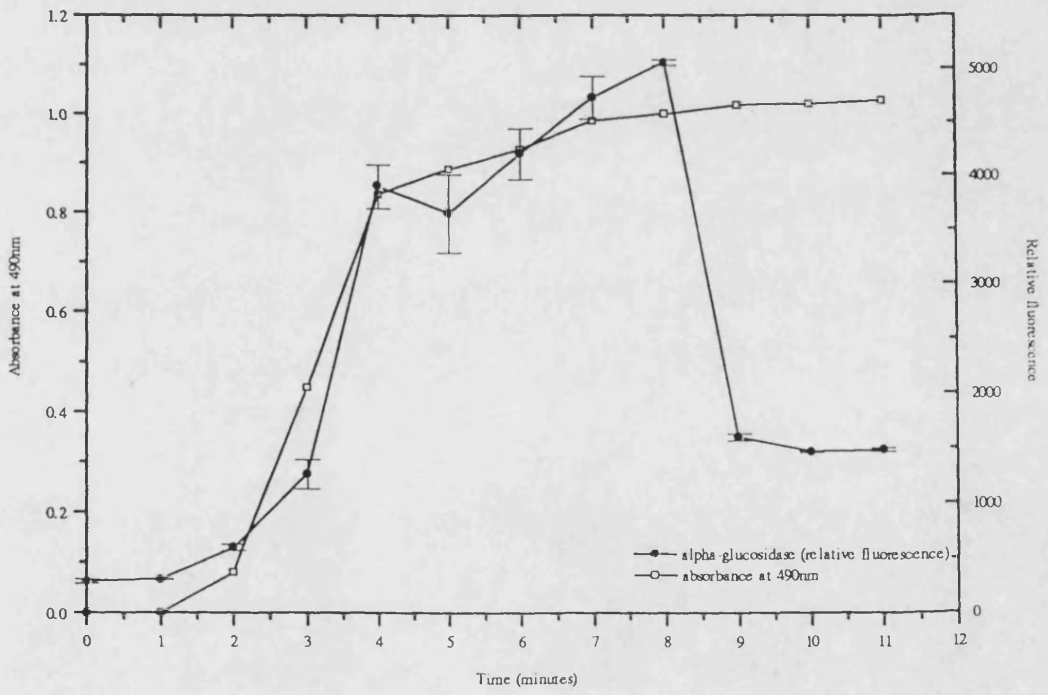
One hundred microlitres of crude spore extract of *B.stearothermophilus* ATCC 7953 (0.03mg/ml protein) was incubated in a 10mM solution of the appropriate carbohydrate in phosphate buffer pH 7.4 for 30 minutes at 60°C. A sample of each reaction mixture was subjected to 4-6 ascents of ascending paper chromatography (Whatman 3M filter paper) using *n*-butanol / pyridine / water (6:4:3), by the method of Welker and Campbell [149]. The chromatogram was removed and air-dried. Reducing sugars were visualised using a modified silver dip method [150]. Solution A was prepared by diluting 1.0ml of saturated silver nitrate to 6.0ml with water, and then to 200ml with acetone. Solution B consisted of 20% of a solution of 10% sodium hydroxide, and 80% methanol. Solution C was an aqueous solution of 0.5M NaS_2O_3. The dried chromatogram was dipped into solution A and allowed to air dry. It was then dipped into solution B until black spots appeared. The chromatogram was next washed with distilled water, and placed in solution C until background coloration disappeared. The chromatogram was rinsed with distilled water.

3.3 Experimental

3.3.1 Production of α-glucosidase during vegetative cell growth

One millilitre of stock vegetative cell culture was used to inoculate 75ml of pre-warmed TSB in a 250ml conical flask. The culture was incubated overnight at 60°C in a shaking incubator. One millilitre of this culture was used to inoculate another flask of pre-warmed TSB.

Figure 3.4. Production of α-glucosidase during vegetative cell growth of *B.stearothermophilus*, ATCC 7953 in BM at 60°C



Samples were taken at 1 hourly intervals, and the absorbance at 490nm, and the relative fluorescence were measured in phosphate buffer pH 7.4 (*3.2.2*) (Figure 3.4).

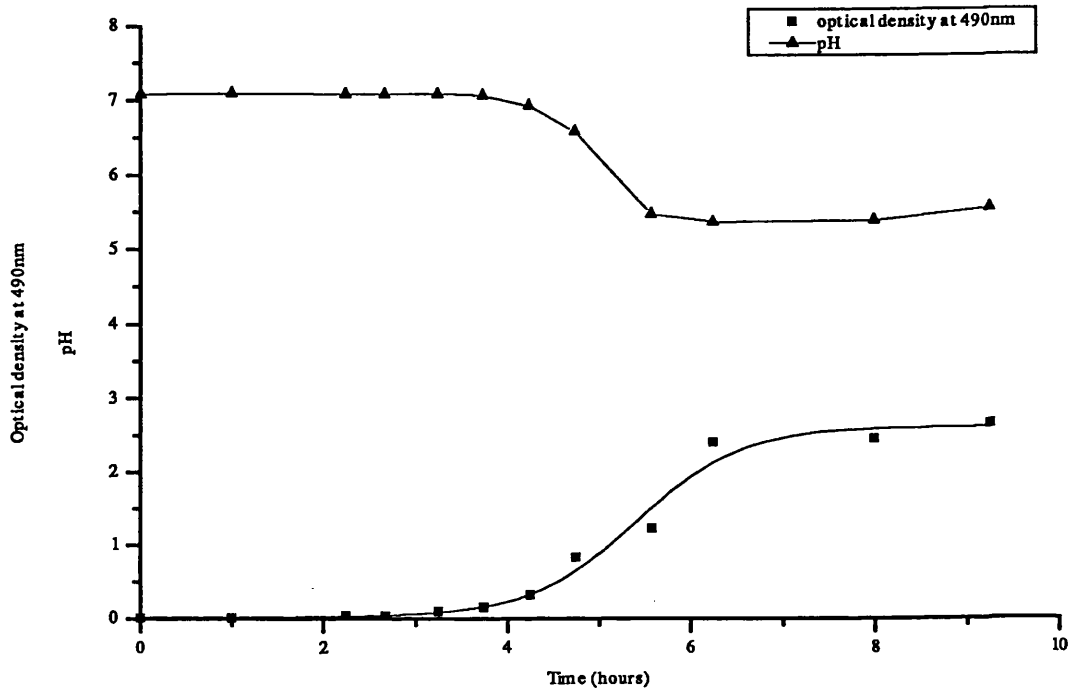
After an initial lag phase at the start of growth, the α-glucosidase activity increased during logarithmic growth of the culture. Activity reached a plateau at the onset of stationary phase (4-5 hours), and the α-glucosidase activity rapidly reduced after 8 hours growth under these experimental conditions.

3.3.2 Change in pH of culture medium during vegetative growth

One millilitre of stock vegetative cell culture was used to inoculate 75ml of pre-warmed TSB in a 250ml conical flask. The culture was incubated overnight at 60°C in a shaking incubator. One millilitre of this culture was used to inoculate another flask of pre-warmed TSB. The culture was incubated at 60°C in a shaking incubator. A 100µl sample was removed at hourly intervals, added to 50µl of 4-MUG solution and 50µl of phosphate buffer pH 7.4, and the fluoresence of the sample was determined (*3.2.2*). The optical density of the vegetative cell culture was determined at hourly intervals to assess the stage of growth. The α-glucosidase activity during vegetative cell growth is shown in Figure 3.5. The culture was incubated at 60°C in a shaking incubator, and samples were removed at pre-determined time intervals. The optical density (490nm) and the pH of the samples were determined (Figure 3.5).

The pH of the culture medium dropped during mid-logarithmic phase, since the buffering capacity of the TSB was poor. The minimum pH attained during batch cultivation was at the onset of stationary phase, the minimum pH reached being pH 5.4. The pH of the culture medium slowly increased to approximately pH 7.0 following incubation of up to 24 hours (not shown). The activity of α-glucosidase at pH 5.4 was less than 15% of the activity at the initial pH of the medium (pH 7.1). During logarithmic growth, the pH of the medium dropped as acid from the metabolism of carbohydrates by the actively growing cells was produced.

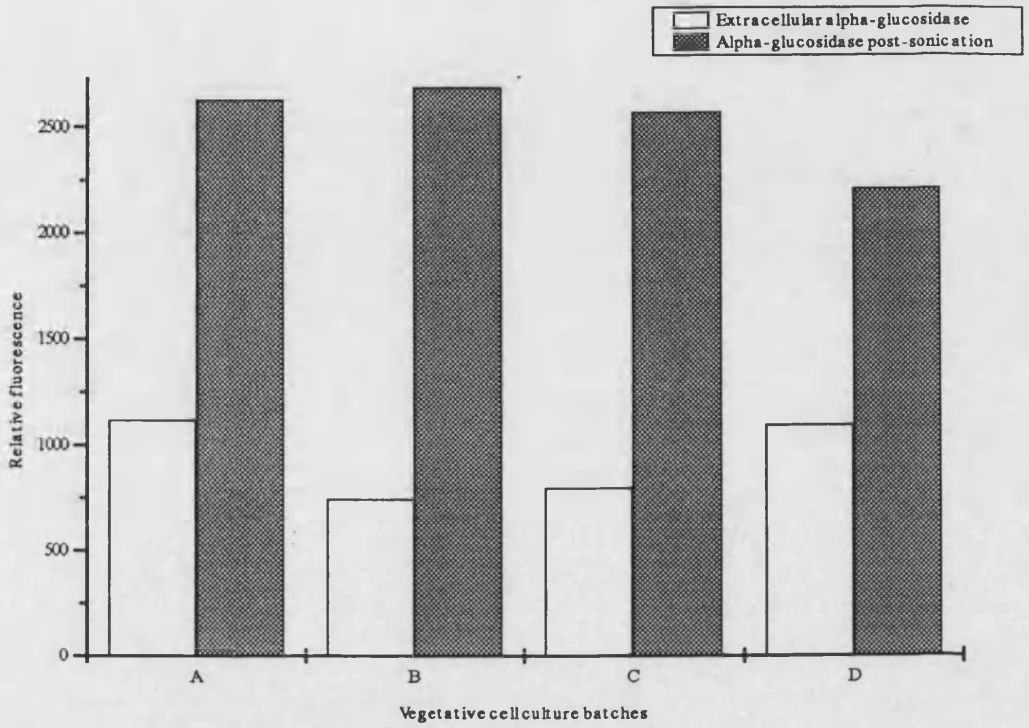
Figure 3.5. Change in pH of culture medium during phases of vegetative growth in batch cultivation of *B.stearothermophilus*, ATCC 7953, in TSB at 60°C



3.3.3 Reproducibility of extraction of α-glucosidase by sonication

Four batches of 6 hour vegetative cell cultures were produced. The extracellular α-glucosidase was assayed immediately using the MUG assay (*3.2.2*) and the total protein content was determined (**2.6**). The cell cultures were sonicated on ice for 1 minute, then centrifuged at 4000rpm for 20 minutes.

Figure 3.6. Alpha-glucosidase levels (per mg protein)` in extracellular medium and after sonication in 4 vegetative cell cultures

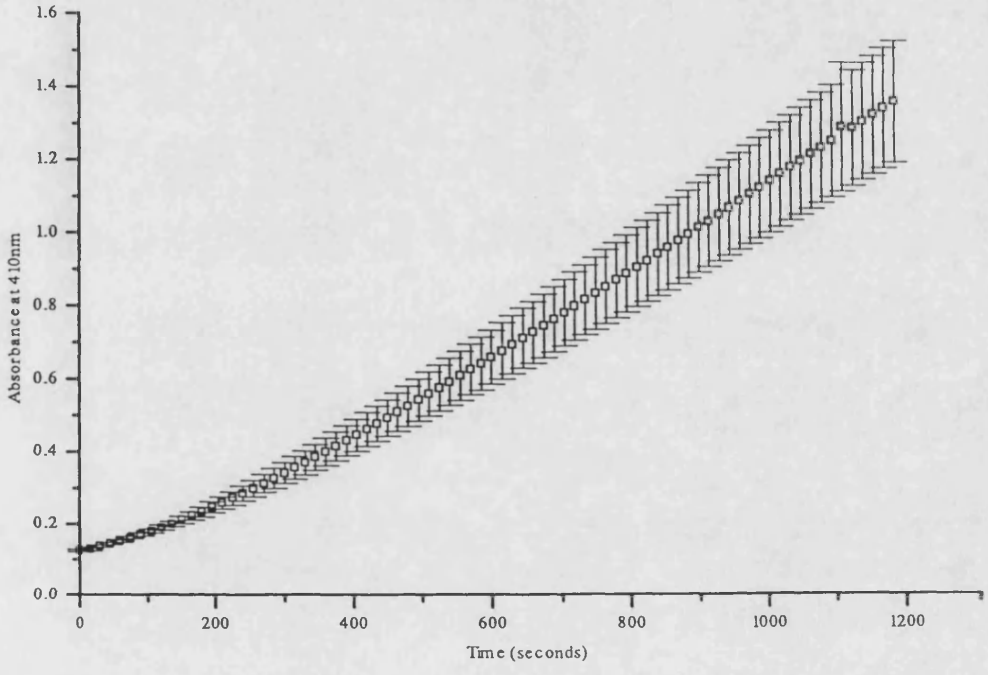


The supernatant was assayed for α-glucosidase activity and total protein content . The results are shown in Figure 3.6. The extracellular α-glucosidase activity (per mg protein) of the 4 separate batches of vegetative cells had a standard deviation of 20.86%. The α-glucosidase activity of the supernatant of the sonicate had a standard deviation of 7.55%. The results of the protein assay showed that there was a large deviation in total protein extracted by the same sonication conditions (14.55%).

3.3.4 Reproducibility of the PNPG Assay

Five replicates of vegetative cell extract (3.75×10^{-4} mg protein) were assayed for α-glucosidase activity (*3.2.2*) in 5mM PNPG, phosphate/EDTA buffer pH 7.4 at 60°C.

Figure 3.7. Reproducibility of *p*-nitrophenyl-α-D-glucoside (PNPG) assay. Five replicates of vegetative cell extract (3.75×10^{-4} mg protein) were assayed with 5mM PNPG in phosphate / EDTA buffer pH 7.4 at 60°C.

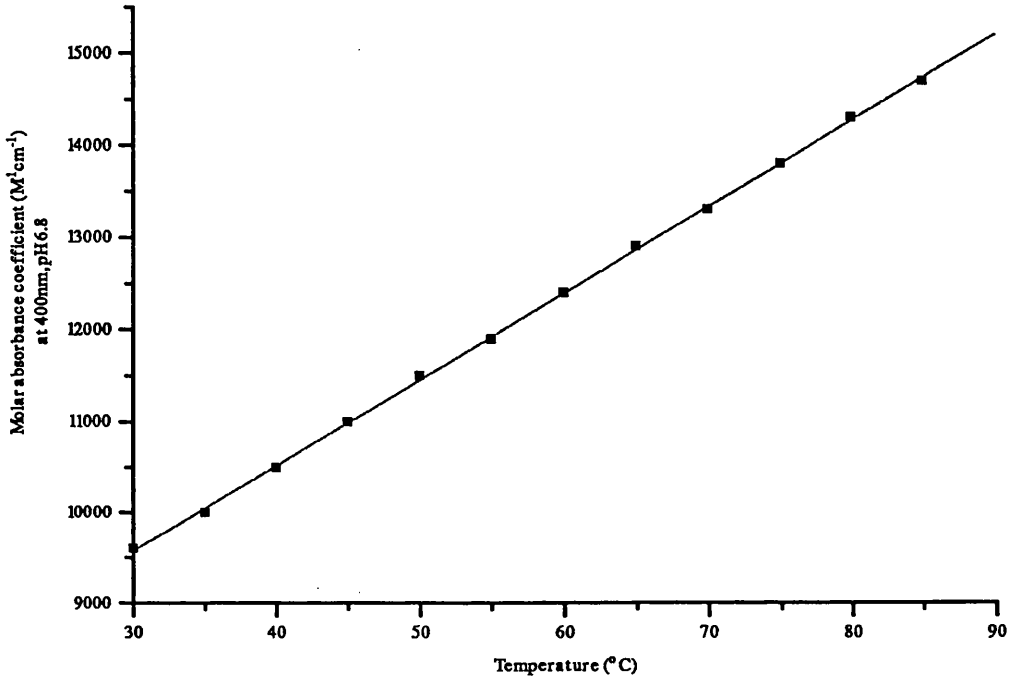


The mean absorbance and the standard error of the reaction mixtures was determined over a 20 minutes period. There was an initial lag phase, probably due to equilibration of the reaction mixture to the temperature of the heat-controlled chamber after addition of the spore extract (stored at 4°C). After this period, the reaction followed a linear profile over the 20 minute period measured (Figure 3.7).

3.3.5 Effect of temperature on the absorbance coefficient of PNP

Figure 3.8. Effect of temperature on molar absorbance coefficient of PNP

From Suzuki, Y, Yuki, T, Kishigami, T, and Abe, S. 1976. Biochim. Biophys. Acta. 445; 386-397 [135].



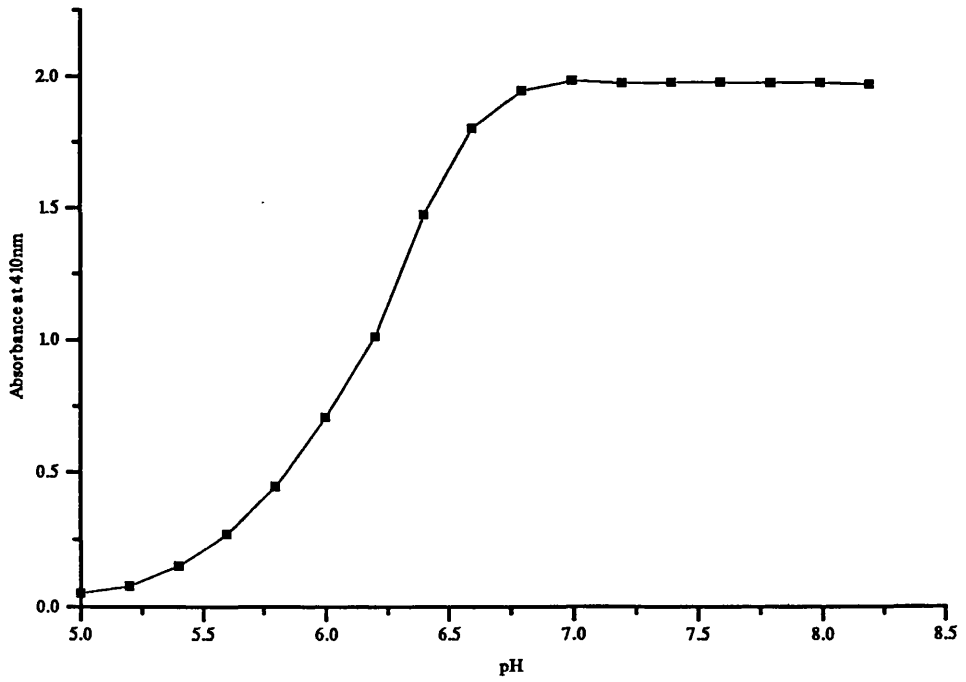
Equation of the best fit straight line $y = 6766.2 + 93.78x$

Figure 3.8 shows the effect of temperature on the molar absorbance coefficient of PNP [135]. The molar absorbance coefficient and the temperature are directly proportional, allowing the molar absorbance coefficient ot be calculated at any temperature between 30°C and 90°C, using the equation of the best fit straight line.

3.3.6 Effect of pH on the absorbance coefficient of p-nitrophenol (PNP)

A stock solution of 10mM PNP solution was prepared in distilled water. Fifty microlitres of the stock solution of PNP was diluted to 1.0ml with a range of Sørensen's phosphate buffers pH 5.0-8.2.

Figure 3.9. Effect of pH on the absolute absorbance of a 1mM solution of PNP



The absorbance of each solution was measured at 410nm at 20°C. Figure 3.9 shows the effect of pH on the absorbance of PNP. It is evident that the absorbance of the solution of PNP increases rapidly between pH 5.0 and 7.0. Changing the pH between pH 7.0 and 8.2, has no effect on the absorbance of the solution. Therefore, when calculating the α-glucosidase activity at different pH values, if the pH at which the

activity is determined is carried out at a pH lower than 7.0, it is necessary for an adjustment of the molar absorbance coefficient for PNP to be made.

3.3.7 Effect of enzyme concentration on the initial reaction rate

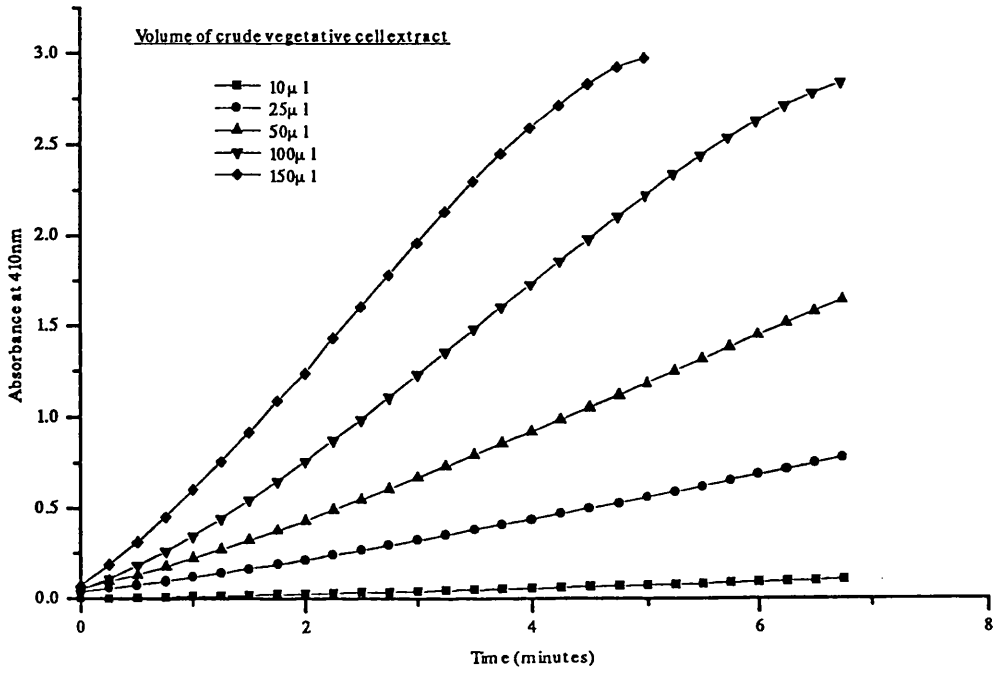
This experiment was carried out to ensure that the reaction followed Michaelis-Menten kinetics, the initial reaction rate being proportional to the enzyme concentration, and to choose the most suitable enzyme concentration for the subsequent experiments.

The absorbance of reaction mixtures at 410nm, at pH 7.4, containing 5mM PNPG in the reaction mixture, and varying amounts of enzyme was measured automatically at 15 second intervals at 55°C. Concentrations of crude vegetative cell extract between 3.75×10^{-5} and 5.6×10^{-4} mg/ml protein were used (10 -150 µl of extract, 3×10^{-3} mg/ml protein).

Figure 3.10 shows the relationship between enzyme concentration and the initial reaction rate. Initially the reaction rate proceeded linearly. A two-fold increase in enzyme concentration led to an approximate doubling of the initial reaction rate. From these data it was decided to use 3.75×10^{-4} mg/ml protein (50µl of 3×10^{-3} mg/ml protein in reaction mixture) of crude vegetative cell extract in subsequent determinations, to allow easy determination of the reaction velocity over the entire time course of the reaction.

Figure 3.10. Effect of enzyme concentration on the initial reaction rate.

Reaction was carried out with crude vegetative cell extract (3 x 10^{-3} mg/ml protein), ATCC 7953, 5mM PNPG, pH 7.4 at 55°C.



3.3.8 Determination of kinetic parameters of vegetative cell α-glucosidase

The reaction was carried out using either 1.875 x 10^{-4} or 3.75 x 10^{-4} mg/ml protein of the crude vegetative cell extract at pH 7.4 at 62°C. The α-glucosidase activity was determined at a range of substrate concentrations; 0.2mM, 0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM and 5.0mM PNPG solution. The results were analysed by the computer direct linear plot method to calculate values of K_m and V_{max} (Table 3.3).

Table 3.3. Kinetic parameters, K_m and V_{max}, for vegetative cell α-glucosidase in crude extract. Parameters are stated as the mean ± standard deviation.

Concentration of crude vegetative cell extract (mg/ml protein)	*K_m (mM)*	*V_{max} (mol PNP/min/mg protein)*
1.875×10^{-4}	1.425 ± 0.275	0.01226 ± 0.00104
3.75×10^{-4}	1.405 ± 0.095	0.01119 ± 0.00003

The mean and standard deviation of the determinations at different enzyme concentrations are K_m = 1.415 ± 0.01 mM (sd = 7.07%); V_{max} = 0.01173 ± 0.00053 mol PNP/min/mg protein (sd = 4.52%).

3.3.9 Determination of kinetic parameters of spore α-glucosidase

The reaction was carried out using crude spore extract, (3.75×10^{-4} mg/ml protein) in phosphate/EDTA buffer pH 7.4 at 62°C. The α-glucosidase activity was assayed with a range of substrate concentrations; 0.2mM, 0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM and 5.0mM PNPG solution. The results were analysed using the manual and the computer direct linear plot methods to calculate values of K_m and V_{max} (Table 3.4). A comparison of the results from the two methods of determination was made.

Table 3.4. Kinetic parameters, K_m and V_{max}, for spore α-glucosidase in crude extract. Parameters are stated as the mean ± standard deviation.

	K_m (mM)	V_{max} (mol PNP/min/mg protein)
Computer method	1.44 ± 0.09	0.00046 ± 0.00004
Manual method	1.625 ± 0.175	0.00047 ± 0.000003

The coefficient of variations of K_m and V_{max} determined by the computer and manual method were 6.04% and 1.28% respectively.

3.3.10 Effect of pH on α-glucosidase activity.

The crude vegetative cell extract and crude spore extract (3.75×10^{-4} mg protein) was assayed for α-glucosidase activity in Sørensen's phosphate buffers of pH between 5.0 and 8.2, with 5mM PNPG at 60°C. The optimum pH for vegetative enzyme activity was approximately pH 7.8 (Figure 3.11).

3.3.11 Effect of pH on α-glucosidase stability.

The crude vegetative cell extract and crude spore extract (3×10^{-3} mg protein) were diluted 1 in 10 in each of the Sørensen's phosphate buffers (0.1ml of vegetative cell extract + 0.9ml of buffer). The mixtures were incubated at 60°C for 30 minutes,then buffered PNPG at pH 7.4 was added to give a final concentration of 5mM PNPG in the reaction mixture. The α-glucosidase activity was measured at 60°C. The optimum pH for enzyme stability was approximately 7.6 (Figure 3.12).

Figure 3.11. Effect of pH on the reaction velocity of α-glucosidase. Reaction was carried out using crude cell or spore extract of *B.stearothermophilus*, ATCC 7953, (3.75×10^{-4} mg/ml protein), 5mM PNPG at 60°C.

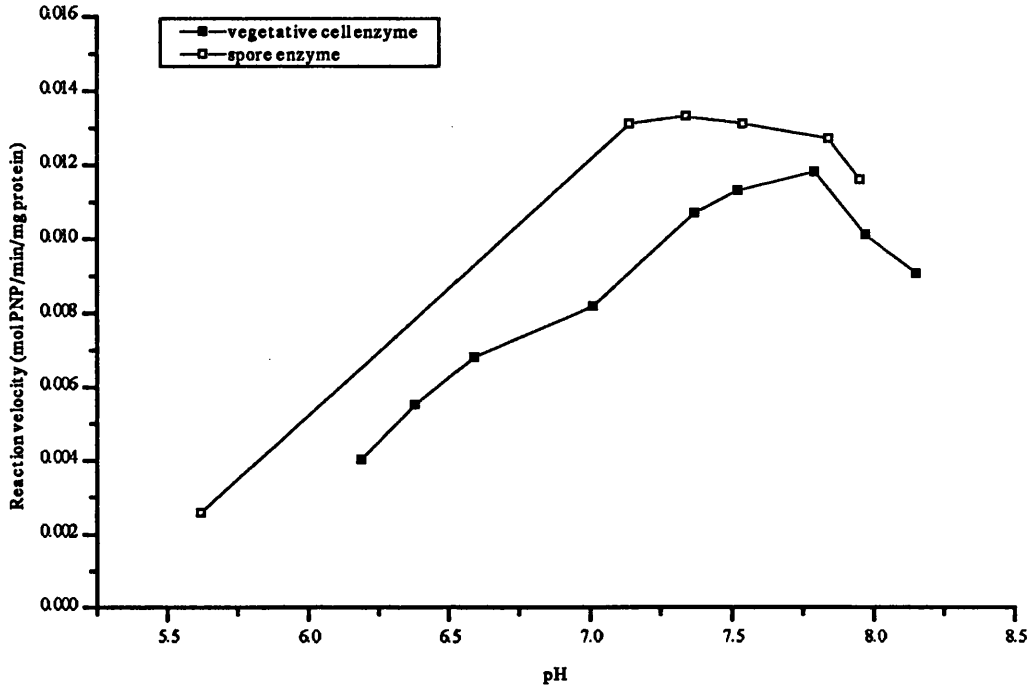
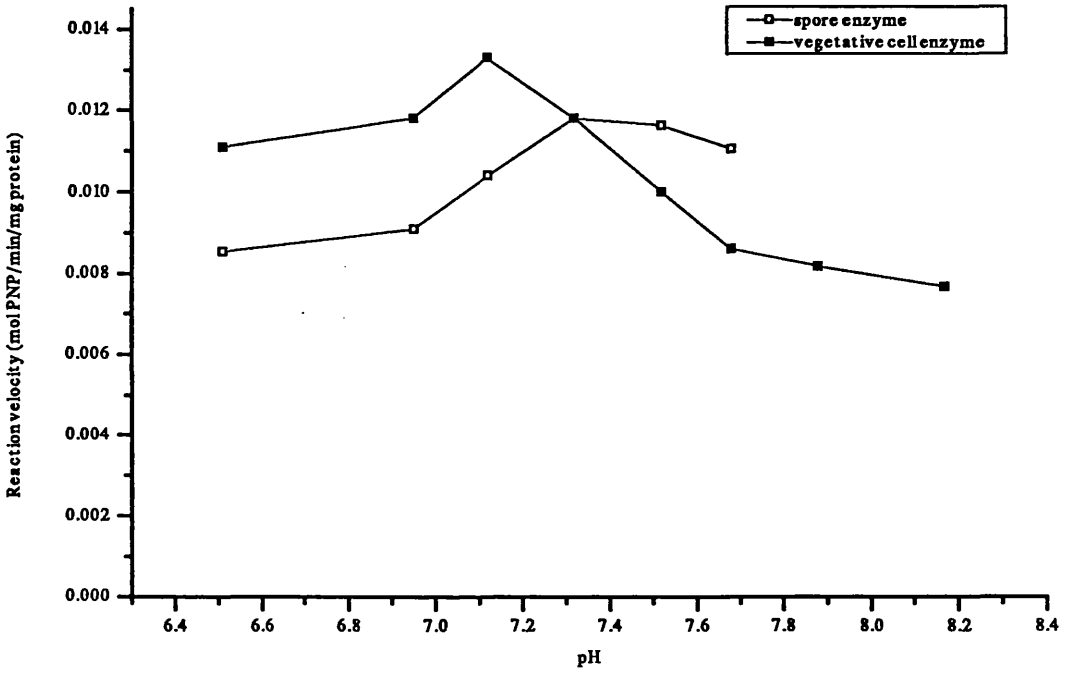


Figure 3.12. Effect of pH on the stability of α glucosidase. Reaction velocity (at pH 7.4, 60°C, 5mM PNPG) was determined after incubation for 30 minutes at 60°C at a series of pH values. Crude vegetative cell and spore extracts of *B.stearothermophilus*, ATCC 7953 were used (3.75 x 10^{-4} mg/ml protein).

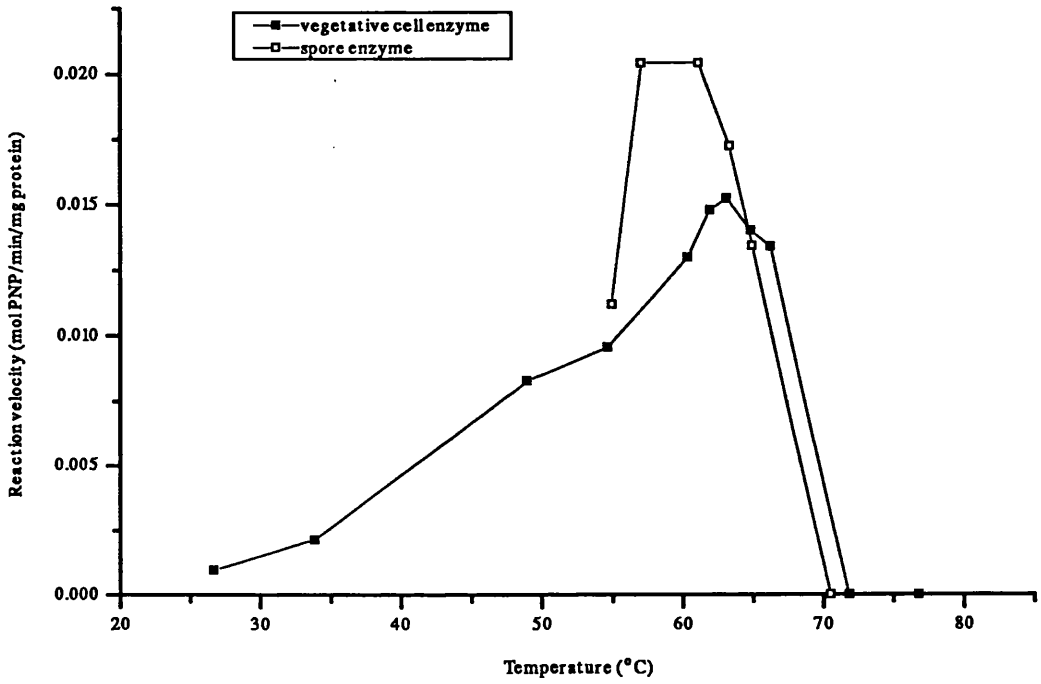


3.3.12 Effect of temperature on α-glucosidase activity.

The α-glucosidase activity of crude vegetative cell extract and crude spore extract (3.75 x 10^{-4} mg protein) was determined at a series of temperatures with 5mM PNPG at pH 7.4 (Figure 3.13). The activity of the enzyme increased rapidly from 20°C, reaching an optimum activity at approximately 63 °C. The α-glucosidase activity dropped sharply at temperatures between 63°C and 77 °C.

Figure 3.13. Effect of temperature on the reaction velocity of α-glucosidase.

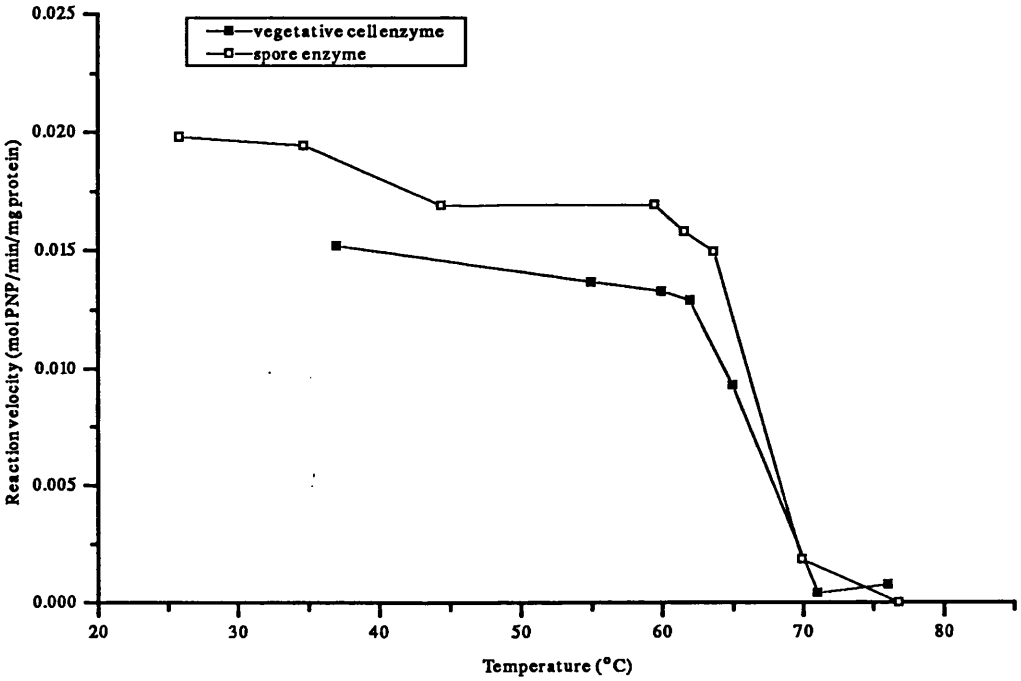
Reaction was carried out with crude vegetative cell or spore extract of *B.stearothermophilus*, ATCC 7953, (3.75 x 10^{-4} mg/ml protein), 5mM PNPG at pH 7.4.



3.3.13 Effect of temperature on α-glucosidase stability.

The crude vegetative cell extract and crude spore extract were incubated in phosphate/EDTA buffer pH 7.4 for 30 minutes at a series of temperatures. The α-glucosidase activity of the extract (3.75 x 10^{-4} mg protein) was determined at 62°C at pH 7.4 with 5mM PNPG (Figure 3.14).

Figure 3.14. Effect of temperature on stability of α-glucosidase. Reaction velocity (pH 7.4, 60°C, 5mM PNPG) was determined after incubation for 30 minutes at a series of temperatures. Crude extracts of vegetative cells and spores of *B.stearothermophilus*, ATCC 7953 (3.75 x 10^{-4} mg/ml protein) were used.



3.3.14 Substrate specificity of spore α-glucosidase

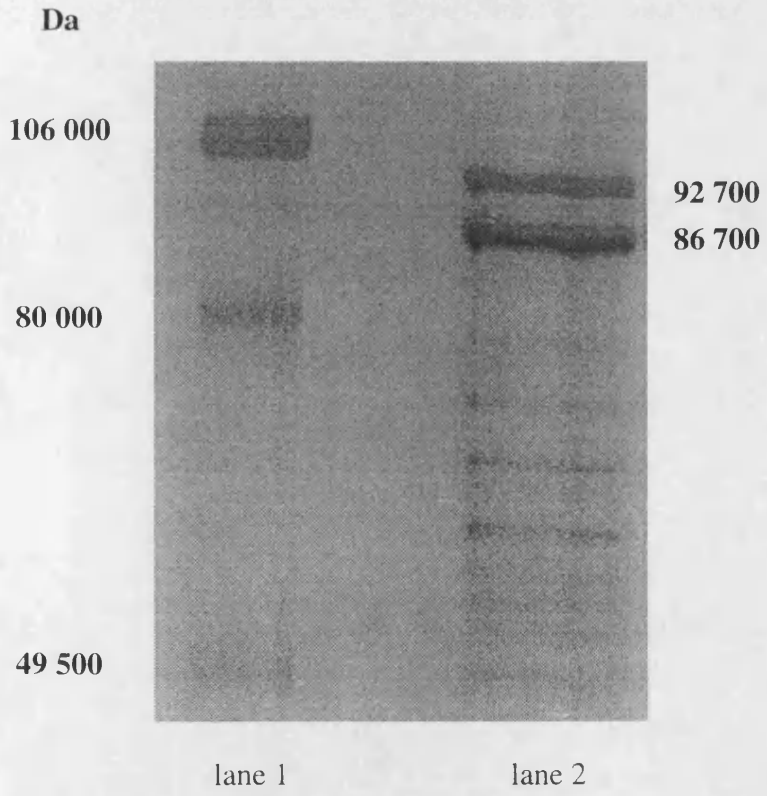
Solutions (10mM) of the following carbohydrates were prepared : maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, nigerose, trehalose, isomaltose, sucrose and glucose. The solutions were incubated with crude spore extract and the reaction mixtures subjected to chromatographic analysis (*3.2.7*). The glucose solution was used as a standard to compare the position of the reaction products on the chromatographic paper.

Glucose was produced by reaction of α-glucosidase with maltose and the maltooligosaccharides (α-1,4). A small amount of glucose was detected following 30 minutes incubation with nigerose (α-1,3) and trehalose (α-1,1). However, no glucose was formed by incubation with sucrose (α-1,2) or isomaltose (α-1,6).

3.3.15 Molecular Weight Determination of α -glucosidase

3.3.15.1 SDS-PAGE

Figure 3.15. 12.5% SDS-PAGE gel of a crude spore extract of *B.stearothermophilus*, ATCC 7953, for molecular weight determination



lane 1 low range molecular weight markers

lane 2 crude spore extract

An SDS-PAGE minigel system (Pharmacia-Biotech, Uppsala, Sweden) was used to separate the proteins in the spore and vegetative cell extracts, and by running molecular weight markers on the gel, it was possible to determine the molecular weight of bands in the crude extracts. A sample of crude spore extract was subjected to electrophoresis on a 12.5% SDS gel (*3.2.3*).

Figure 3.15 shows the SDS-PAGE gel of the molecular weight markers and the bands of proteins found in the crude spore extract of *B.stearothermophilus*, ATCC 7953. From this gel the molecular weight of the two major proteins were determined to be 92 700 and 86 700.

3.3.15.2 Gel Filtration

The values of K_{av} determined for the marker proteins (Table 3.5) were used to produce the calibration plot, relating molecular weight to K_{av} (Figure 3.16).

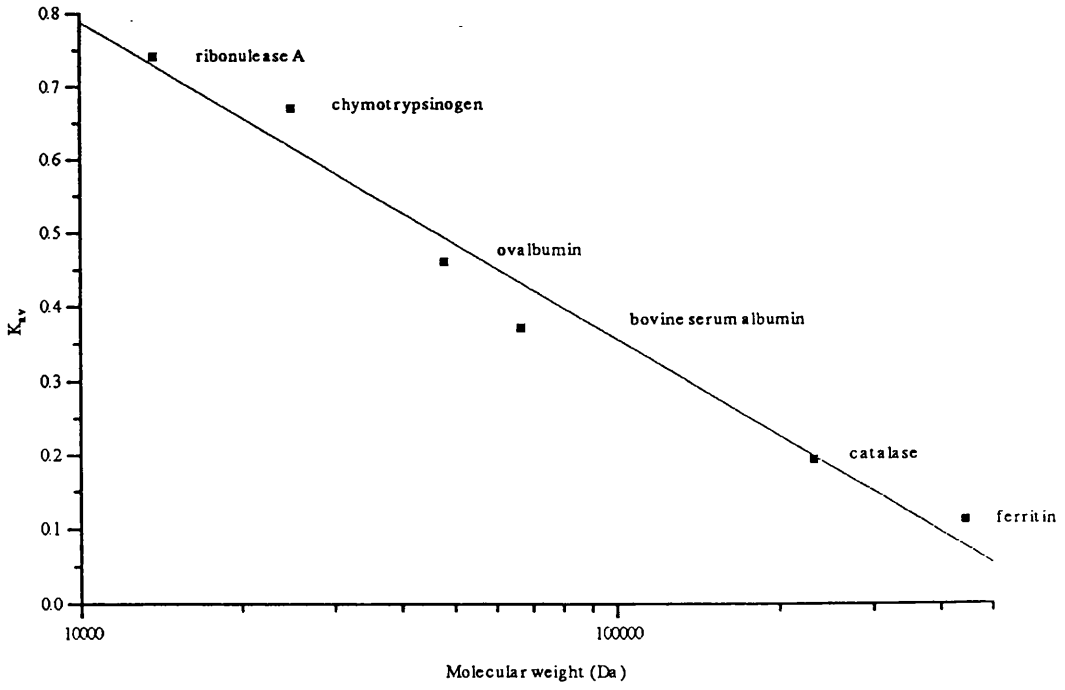
Table 3.5. Molecular weight markers used for calibration of Sephadex G-200 column

Marker	*Concentration of sample for injection (mg/ml)*	*Molecular weight*	*Elution volume V_e (ml)*	K_{av}
Ferritin	0.5	440 000	139.60	0.112
Catalase	5	232 000	163.60	0.193
Bovine serum albumin	7	67 000	215.95	0.372
Ovalbumin	7	43 000	242.12	0.461
Chymotrypsinogen	3	25 000	287.93	0.618
Ribonuclease A	10	13 700	316.29	0.714

The elution volume of the α-glucosidase from the column was 211.59ml, which correlated with a K_{av} value of 0.357. The molecular weight of the α-glucosidase was calculated from the calibration plot to be 100 040.

Figure 3.16. Calibration of Sephadex G-200 gel filtration column with molecular weight markers

V_o = 400ml
Column void volume (elution volume for blue dextran) = 106.89ml
Sample volume = 0.5ml
Flow rate = 0.55ml / min
Eluent : Sorensen's phosphate buffer pH 7.4



Equation of the best fit straight line $y = 2.51638 - 0.43182\,x$

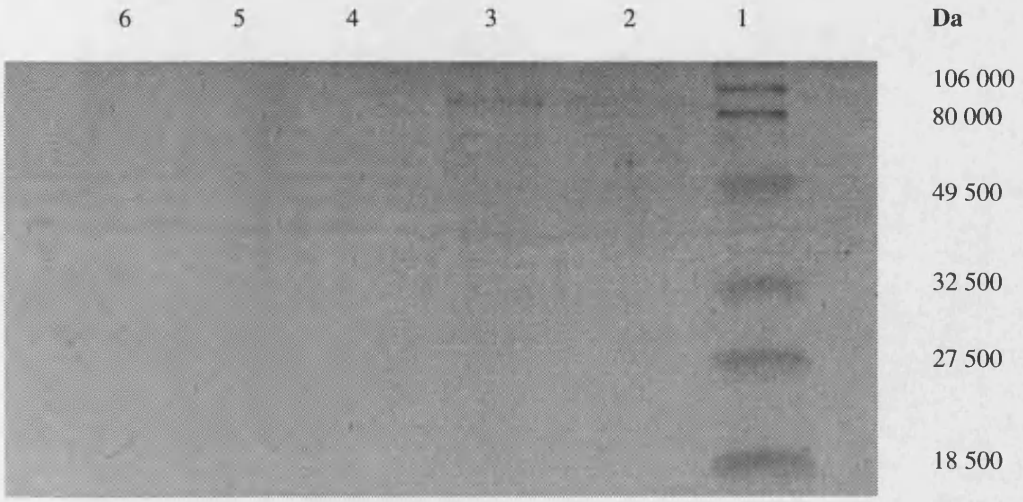
3.3.16 SDS-PAGE of spore extracts of different Bacillus *species*

SDS-PAGE was carried out on sonicates of several spore suspensions of *Bacillus* species, to compare the occurence of alpha-glucosidase in *Bacillus stearothermophilus* with that in other species, or under different growth conditions. The following spore suspensions were used:

1. *B.stearothermophilus*, ATCC 7953, grown on 3M sporulation medium
2. *B.stearothermophilus*, ATCC 7953, inoculum medium, TSB + 0.5% maltose, sporulation agar, 3M + 0.5% maltose
3. *B.stearothermophilus*, NCIMB 8224
4. *B.subtilis* NCTC 10073
5. *B.coagulans* (DJ, 3M)

Figure 3.17. 12.5% SDS-PAGE gel of crude spore extracts of different *Bacillus* species.

1. LMW markers, 2. ATCC 7953 (3M), 3. ATCC 7953 (maltose-induced), 4. NCIMB 8224, 5. NCTC 10073, 6. *B.coagulans*.



The spore suspensions were diluted to give 10ml of a total count of 1 x 10^8 / ml prior to the production of spore extracts. Each spore suspension was then sonicated for 5 minutes on ice. The spore sonicates were centrifuged for 20 minutes at 4000rpm. The supernatant was concentrated to 3ml with an Amicon Ultrafiltration unit with a 10kD membrane. The spore extracts were run on a 12.5% SDS-PAGE gel and stained (*3.2.3*) (Figure 3.17).

The presence of a protein of approximately 92,000D can be seen in lanes 2 and 3, but is absent from the other extracts. This band corresponds to the position of alpha-glucosidase, as determined by previously run SDS-PAGE. The alpha-glucosidase band is much bolder in the extract of *B.stearothermophilus* that was grown in maltose-supplemented medium, suggesting that this medium encourages production or expression of higher levels of the enzyme. The other species do not contain a band at this molecular weight. This could be interpreted as the absence of alpha-glucosidase from sites within the spore where sonication can cause release, or the modification of the enzyme compared to thermophilic species, or simply a significantly lower level, thus not visible on the gel. There are several faint bands present between 40kD and 80kD, of proteins that are consistently present in all the spore extracts.

3.4 Discussion

Various characteristics of the vegetative cell and spore-associated alpha-glucosidases were studied. A comparison of the results of characterisation experiments carried out on the two crude extracts was made. The properties of the α-glucosidase of *B.stearothermophilus* ATCC 7953 were compared with similar enzymes that have been previously characterised [124].

The method of enzyme extraction, sonication, was used to minimise the disruption to alpha-glucosidase activity, which is substantial when extraction methods relying on enzymes and chemicals are employed. There was a large standard deviation of the amount of total protein and the alpha-glucosidase that was extracted from spores or vegetative cells by sonication. This is likely to be due to the nature of the process itself, as well as reflecting different levels of the enzyme in individual spores. Electron microscopic evidence suggests that individual spores have vastly different responses to a sonication process, possible due to differing states of dormancy and resistance of the spores in a given population. At least a two-fold increase in alpha-glucosidase activity was achieved by sonication of the vegetative cell cultures, compared with the alpha-glucosidase concentration found in the extracellular medium.

In most cases it is preferable to study an enzyme in a state as close to its natural condition as possible. Any purification process would remove any substances that may have an effect on the activity of the enzyme within the spore. The results of a K_m and V_{max} determination of the purified enzyme could be compared with that of the crude extract to assess whether any inhibitory or stimulatory compounds were present, and their effect on enzyme activity.

For display purposes, the Lineweaver-Burk plot is the best known and simplest method. The reciprocal values of K_m and V_{max} are indicated directly by the intercepts

on the 1/s and 1/v axes respectively. Figure 3.20(a) shows the distortion of experimental errors caused by the double reciprocal plot. Also, the data points are closely spaced near to the 1/v axis, and are widely spaced at higher values. This makes it difficult to estimate the $1/K_m$ value by extrapolation of the line to the 1/s axis, although this can be compensated for by intermediate data points. The Hanes plot is preferred to the Eadie-Hoftsee plot because the distortion of the error bars is minimal with this plot (Figure 3.20(b) an 3.20(c)). In the Eadie-Hoftsee plot, the dependent variable occurs on both axes, and thus the errors in the *x* and *y* axes are interdependent. This hinders fitting the best line to the data. When a comparison of the 3 plots was made using computer-simulated data, with random error included to account for experimental error, the Lineweaver-Burk plot gave the least accurate estimates of K_m and V_{max}. The Hanes plot is therefore the preferable graphical method to use to examine the data visually, and indicate whether the data follow a linear profile, and that the error is small and randomly-distributed [145, 146]. It is always necessary to plot a graph to visually inspect data to ensure that the results adhere to any assumptions inherent in the analysis, even when a computerised calculation of kinetic parameters is to be used. However, the only graphical method that should be used to calculate kinetic parameters for publication is the Direct Linear Plot [145, 147] (Figure 3.19). The Hanes plot was therefore used for the initial inspection of the data, and the Direct Linear Plot (both manual and computerised methods) was used for determination of the values of K_m and V_{max} [145, 147].

Figure 3.19. The direct linear plot of data exactly fitting the Michaelis-Menten equation. From Henderson, P.J.F., Statistical analysis of enzyme kinetic data. Enzyme Assays. A Practical Approach. 1992. Ed. Eisenthal, R and Danson, M.J. Oxford University Press. New York [145].

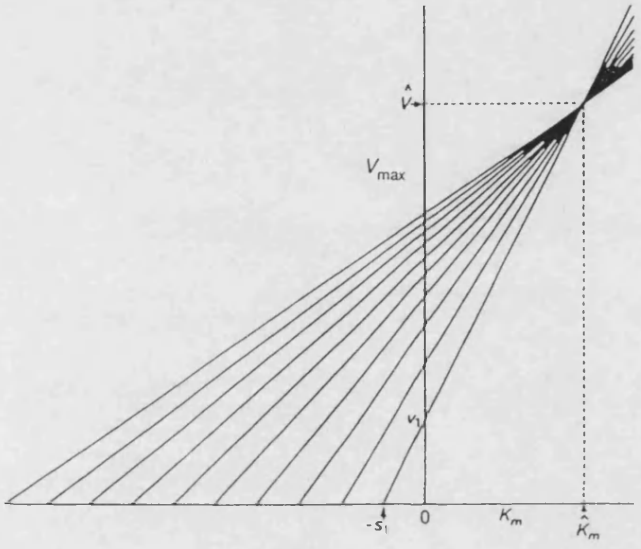
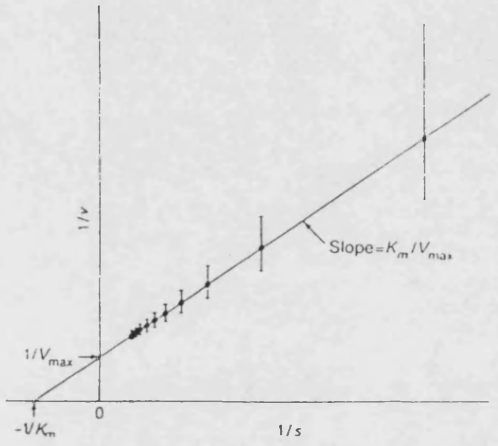


Figure 3.20(a). Lineweaver-Burk plot (1/v against 1/s) [145]. The error bars show the distortion caused by the double reciprocal plot, of errors of a size of $\pm 0.05\ V_{max}$.

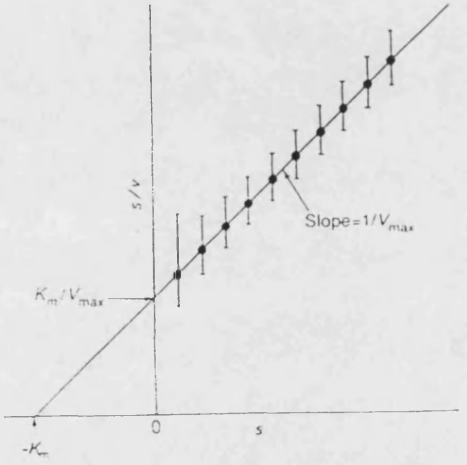
(b). Hanes plot (s/v against s). Error bars $\pm 0.05\ V_{max}$.

(c). Eadie-Hoftsee plot (v against v/s). Error bars $\pm 0.05\ V_{max}$.

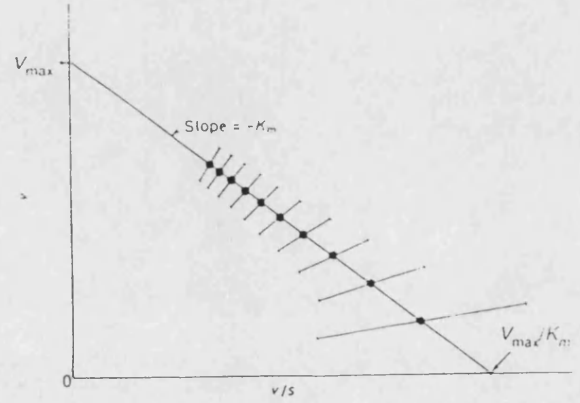
(a)



(b)



(c)



Determination of K_m and V_{max} is now commonly carried out using a computer, eliminating the error associated with visual inspection of data. The least-squares fit of the data to a hyperbola of v against s is the process used to calculate the kinetic parameters and their standard deviation. There are several assumptions in the use of the least-squares fit method that are difficult to verify, such as the correct weighting scheme, and errors in the 2 variables being independent of each other. These factors do not pose problems in the direct linear plot. However, the overriding advantage of the computerised least-squares fit method is that multi-substrate systems, and the effect of activators and inhibitors can be accounted for when using this method. These factors were not applicable in this work, and therefore the Direct Linear Plot was the most suitable method.

Using the crude extracts, the determinations of K_m and V_{max} are of the same order of magnitude as published data for other bacterial alpha-glucosidases [124, 135], in which values of K_m between 0.6mM and 0.9mM PNPG were reported. Any differences may be explained by the difference in methodology in the determination of kinetic parameters, since the majority of the published work was carried out prior to the introduction of the direct linear plot and other modern computerised methods. The Lineweaver-Burk plot continues to be a popular method to use to calculate kinetic parameters despite its disadvantages. The values of K_m were comparable for the spore-derived and vegetative cell forms of the enzyme, being an indirect measure of the affinity of the enzyme for its substrate.

The effects of the varying pH and temperature conditions were essentially similar for the spore and vegetative cell enzymes. The spore alpha-glucosidase generally showed a broader range of temperature and pH maxima than the vegetative cell enzyme. Both forms of the enzyme were stable up to 65°C, above which temperature the stability rapidly decreased. The reactions were initially carried out using vegetative cell extract, since it was more easily produced in large quantities than the spore extract. In some

cases, the reactions carried out with spore extract were carried out over a narrower range of conditionsto conserve the spore extract.

The results presented here correlate with the results of the alpha-glucosidase of *Bacillus thermoglucosidius* KP 1006 [135]. Levels of alpha-glucosidase increased throughout logarithmic growth of *B.stearothermophilus*, ATCC 7953, in TSB or BM at 60°C, and decreased as cessation of growth occured at the onset of stationary phase. During batch cultivation in TSB, the initial pH of the culture medium is approximately 7.3, but this reduced during logarithmic growth of *B.stearothermophilus* to a minimum pH of 5.5 at the end of the logarithmic growth. Alpha-glucosidase is unstable and has low activity at this pH, but during logarithmic growth active protein synthesis allows replacement of inactivated enzyme, so that the harmful effect of the adverse pH conditions is masked. The effect of the low pH is only observed at the start of the stationary phase when active synthesis ceases, and the effect on the pre-existing enzyme is revealed. The activity of non-specific proteases during stationary phase leads to inactivation of many hydrolytic enzymes that were active during logarithmic growth.

The α-glucosidase of *B.stearothermophilus* ATCC 7953 is most similar to the less common group of α-glucosidases which have high activity towards PNPG, and activity towards maltose and malto-oligosaccharides, but little or no activity towards α-1,6 glycosidic bonds, *ie.* isomaltose and isomaltosaccharides [125]. This enzyme, in addition, has low activity towards α-1,3 linkages, but no activity towards α-1,2 glycosidic bonds. These results correlate well with the characteristics of *B.stearothermophilus* 12016, which was studied by Suzuki *et al* [134]. This alpha-glucosidase from *B.stearothermophilus*, ATCC 12016, capable of hydrolysing PNPG was found to have a molecular weight of 47000, and pH and temperature optima of pH 6.3 and 70°C respectively. A K_m value for nitrophenyl-glucoside was 0.63mM. No carbohydrate or cysteine residues were associated with the enzyme. This alpha-

glucosidase had a restricted substrate specificity, hydrolysing glucose from the non-reducing terminal alpha-1,4 bonds of oligomaltosaccharides (2-6 glucose units) and amylose, amylopectin, soluble starch and alpha-limit dextrins. The enzyme was classified as an exo-alpha-1,4-glucosidase [134]. These properties are similar to those presented here. The affinity of the enzyme from *B.stearothermophilus* 12016 for the substrate, PNPG, is greater than that of the α-glucosidase of *B.stearothermophilus* ATCC 7953, although the general characteristics are similar. The latter enzyme also shares similarities with α-glucosidases from *Saccharomyces cerevisiae*, *B.circulans* and other bacilli [124].

Molecular weight determination by SDS-PAGE gave two prominent bands in the crude spore extract, 92 700 and 86 700. The larger band was associated with alpha-glucosidase activity, as shown with staining of a native polyacrylamide gel with PNPG, or from alpha-glucosidase assay of the corresponding gel filtration fraction. The smaller molecular weight band had no alpha-glucosidase activity, but was found to have alpha-amylase activity (Lewis Woodson, 3M Healthcare, St. Paul - personal communication). This concurs with work carried out by Sata *et al* [127], who showed that α-glucosidase of *B.circulans* was found in association with amylase. The two enzymes were susceptible to inhibition by glucose, thus regulating their production. However, the amylase was more sensitive to the regulation than the α-glucosidase, and this provided a means of finely controlling the production of the amylase.

Treatment with SDS generally cleaves proteins into their sub-units, therefore the molecular weight determined by this method may not be the true value if the protein exists in oligomeric form. The molecular weight determined by SDS-PAGE (92 700) was compared with the value determined by the gel filtration method (100 040). The discrepancy between the two values can be accounted for by errors associated with the two methods. The results show that the alpha-glucosidase exists in monomeric form in the spore.

The spore alpha-glucosidase has a molecular weight of 6 000 greater than the vegetative cell form, as determined by the immunolabelling of the two bands on a nitrocellulose membrane. This molecular weight difference is equivalent to approximately 45-50 amino acid residues. It is probable that the two forms of the enzyme have originated from the same genetic code, but that an additional portion codes for the modification to the spore enzyme. This is unusual, since alteration from vegetative cell form to spore form of enzymes often involves proteolysis, resulting in the spore form of the enzyme having a lower molecular weight [113]. This modification is presumably in order to allow specific targeting of the enzyme to its final location within the spore, and to enable transport of the enzyme across the spore coats. Similar differences are observed between the sequences coding for intracellular proteins and proteins that are exported across membranes in bacteria and mammalian cells [151, 152, 153, 154]. A similar mechanism is likely to account for the attachment of enzymes to the outer surfaces of spores, and the export of proteins through the spore coats.

Exported proteins are synthesised as precursors that undergo removal of amino-terminal extensions by proteases [151]. The information necessary to localise and assemble an outer membrane protein is usually in the form of a signal (leader) sequence, although it can be interspersed throughout the primary sequence. The signal sequence generally consists of 18-35 amino acids. There is a positively charged N-terminal, followed by a stretch of hydrophobic residues (H region), and a more polar C region with a consensus cleavage site, Ala-X-Ala, where cleavage occurs after the carboxy-terminal alanine. X is usually a bulky amino acid residue. Occasionally, both the alanine residues can be replaced by other amino acid residues with short side chains [155]. The targeting and attachment to the inner membrane is postulated to be via the a signal peptide-dependent pathway, which comprises *sec* gene products and signal peptidases. Complete transport of proteins across the outer membrane requires several additional accessory proteins. The membrane-triggered folding hypothesis suggests that the N-terminal signal sequence confers a conformation on the precursor protein

that renders it soluble. The interaction of the precursor protein with the membrane triggers a change in conformation causing association with the membrane, which along with the protein motive force leads to translocation of the protein across the membrane. This hypothesis does not necessarily imply that translocation occurs entirely after completion of protein synthesis [156].

The construction of gene fusions for hybrid proteins in which the signal sequence of one protein is replaced by that of another has given valuable insight into protein export in bacteria [157]. The signal sequence of a periplasmic β-lactamase was substituted with that of periplasmic alkaline phosphatase. The alkaline phosphatase was still found localised in the periplasm [158]. The question of whether the signal sequence is purely to initiate the export process, or whether it contains information about the intended location of the protein has been addressed by several studies. Tommassen *et al* [159] found that when the outer membrane porin, PhoE, signal sequence was replaced with that of β-lactamase, it was still properly exported to the outer membrane, suggesting that localisation to the outer membrane involved amino acid sequences or structures within the body of the mature protein, and was not due to the signal sequence alone. In bacterial cells, many cytoplasmic membrane proteins are not synthesised with cleavable amino-terminal sequences. Some proteins are effectively cytoplamsic proteins bound to the membrane by interactions with integral membrane proteins, whilst others contain hydrophobic membrane spanning sections that anchor them firmly to the membrane [157]. A third type of inner membrane proteins contain a cleavable sequence and span the membrane once. It is believed that these proteins are subjected to the usual export process, but remain anchored to the membrane by a carboxy-terminal sequence [160]. Such a mechanism is likely to account for the localisation of alpha-glucosidase to the spore coats (See Chapter 5), and their release into the extracellular medium to act as a scavenging enzyme.

The gene encoding the alpha-glucosidase of *Bacillus* species SAM 1606 has been sequenced [130]. The amino-terminal sequence of 10 amino acid residues of the alpha-glucosidase was determined (Alta Bioscience, University of Birmingham) (Appendix 1). The sequence data were compared with the bacterial DNA databases (EMBL / GenBank / DDBJ) to highlight any sequence homology with other proteins. 71.4% homology over a 7 amino acid overlap was found with the sequence of the alpha-glucosidase of *Bacillus* species SAM 1606. Some similarity was also found with an alpha-amylase gene in *Streptomyces lividans*. Similar intracellular and extracellular sporulation-specific glucan-1,4 glucosidases were found in *Saccharomyces cerevisiae* [161].

From the evidence presented, it seems most likely that the alpha-glucosidase of the spores and vegetative cells is derived from the same genetic sequence, but some modification has occured to equip the spore enzyme for its role and location within the spore. From a pragmatic viewpoint, this would be the most likely option, since the spore is a survival mechanism, which contains molecules required for the initial stages of germination. Therefore the production, and preservation of large amounts of essentially similar DNA would be wasteful. Modification of the transcription of a single DNA sequence for the enzyme, or post-translational modification of the protein would be the preferable mechanism for production of two forms of the same enzyme from the minimal amount of DNA.

Chapter 4

ALPHA-GLUCOSIDASE ACTIVITY DURING SPORE GERMINATION AND VEGETATIVE CELL GROWTH

Chapter 4

ALPHA-GLUCOSIDASE ACTIVITY DURING SPORE GERMINATION AND VEGETATIVE CELL GROWTH

4.1 Introduction

Chapter 3 detailed the characterisation of the vegetative cell and spore-associated alpha-glucosidase enzyme. To study the function of this enzyme in the Attest ™ Rapid Readout Biological Indicator, and to elucidate its role in spores, experiments were carried out to investigate factors controlling its activity, both during spore germination and during vegetative cell growth. Potential methods to improve the readout time and sensitivity of the BI are described.

4.2 Materials and Methods

4.2.1 Preparation of Recovery Medium used in the Rapid Readout Biological Indicator

The following components were dissolved in distilled water and dispensed into 500ml medical flat bottles and autoclaved at 121°C for 15 minutes.

Trypticase peptone	5g/l
Phytone peptone	10g/l
L-Alanine	0.17g/l
Bromocresol purple	0.03g/l
Methylumbelliferyl-α-D-glucoside	0.02g/l

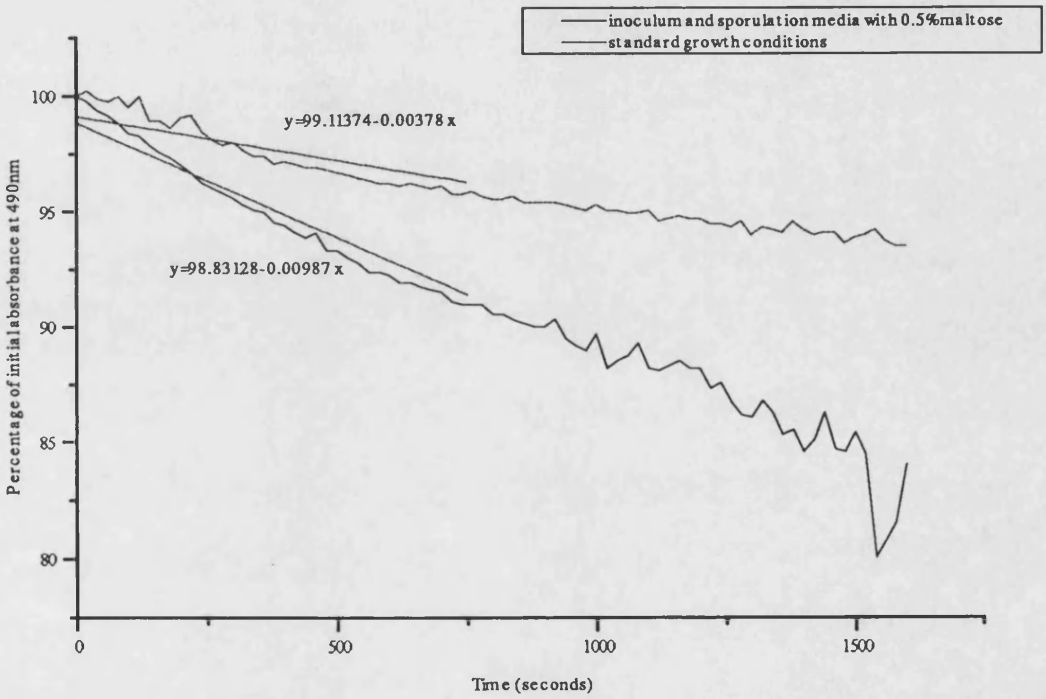
4.2.2 Measurement of germination of spores using optical density method

A volume of spore suspension sufficient to give an optical density at 490nm of between 0.8 and 1.0 absorbance units was added to the germination medium, pre-warmed to the germination temperature, 60°C, to give a final volume of 1.0ml. The mixture was incubated in the temperature-controlled chamber of a Pye-Unicam UV / VIS spectrophotometer, and absorbance measurements were recorded automatically at pre-determined time intervals. A graph of the percentage initial absorbance at 490nm against time was plotted. The effect of various parameters on the rate and extent of germination was studied.

4.3 Experimental

4.3.1 Comparison of germination profile of B.stearothermophilus spores grown under standard conditions and with maltose-supplemented media

Figure 4.1. Germination of *B.stearothermophilus* spores, ATCC 7953, in TSB at 60°C, measured by absorbance at 490nm



The optical density method of estimating germination of spores was used to compare the germination characteristics of spores that were produced using standard conditions (*4.2.2*) with spores that were produced on media that had been supplemented with 0.5% maltose. The germination of the spores was determined in TSB at 60°C (*4.2.2*). The initial germination rate (over 750s) was determined. The initial linear germination rates

over the first 750 seconds of incubation are shown in Figure 4.1. It can be seen that the germination rate for the spores grown on maltose-supplemented media is three times greater than that for spores grown under standard conditions.

Figure 4.2. Germination of *B.stearothermophilus*, ATCC 7953, spores in TSB at 60°C, measured by absorbance at 490nm

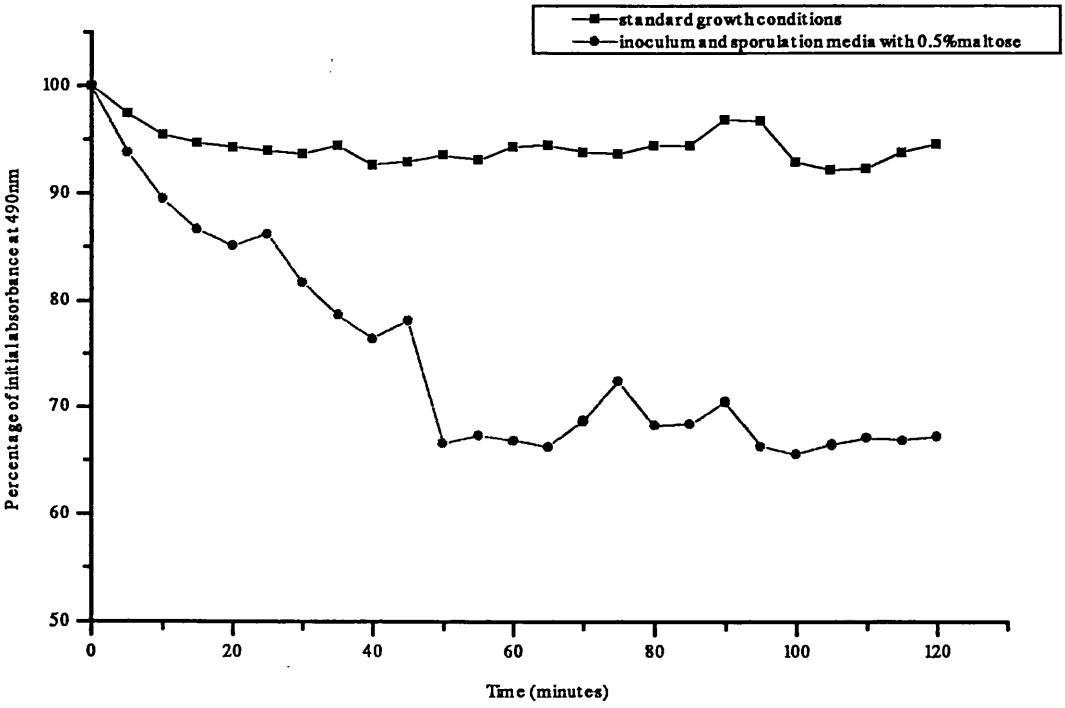


Figure 4.2 shows the continued germination of the spore populations for 2 hours, showing that the extent of germination after this period remains much less for the unsupplemented spores. The optical density of the supplemented spores had reduced by approximately 30% over 2 hours, whereas the optical density of the unsupplemented spores had fallen only by about 7%. It is apparent therefore that the germination of the

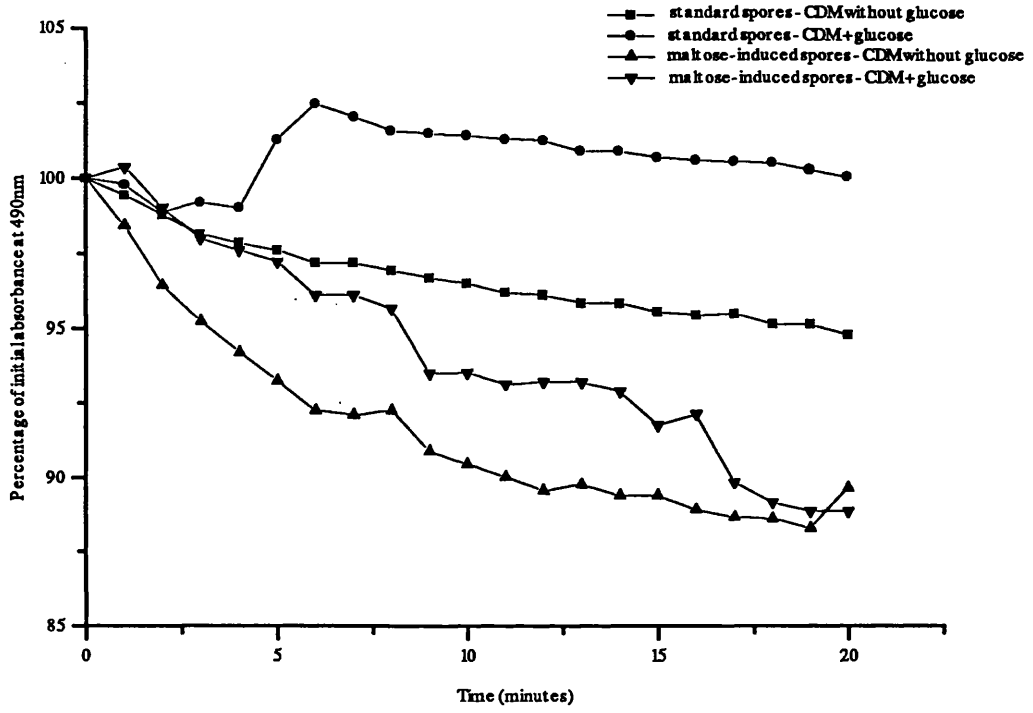
spores is greatly affected by the sporulation conditions of the spores. Spores containing higher levels of alpha-glucosidase seem to germinate to a greater extent and with a faster initial rate than standard spores. This implicates alpha-glucosidase enzyme as having a role in the germination of *B.stearothermophilus*.

4.3.2 Germination of B.stearothermophilus *spores (standard spores and maltose-supplemented spores) in chemically-defined medium (CDM)*

Chemically-defined medium (CDM) was used as a germination medium to allow assessment of the effect of glucose on the rate and extent of germination. Glucose is known to be an inhibitor of alpha-glucosidase at certain concentrations. Spores that had been produced under standard conditions and maltose-supplemented spores were used to examine the effect of glucose on spores that had increased levels of the enzyme.

Figure 4.3 shows the germination profiles of standard and maltose-supplemented spores in CDM in the presence and absence of glucose (7.5mM). The mean absorbance value of 3 replicates were plotted for each data point for clarity and ease of comparison. The standard error of the absorbance measurements was steady throughout the period investigated. The rate and extent of germination was greater in CDM with the maltose-supplemented spores, as occurred in TSB. In both the standard and supplemented spores, germination was more rapid in CDM without glucose. There was an initial increase in optical density of the standard spores in CDM with 7.5mM glucose over the first 5 minutes, after which time the expected reduction in optical density occurred.

Figure 4.3. Germination profiles of standard and maltose-induced spores in CDM at 60°C, in the presence and absence of glucose (7.5mM), measured by absorbance at 490nm



4.3.3 Factors affecting initial germination rate of B.stearothermophilus spores

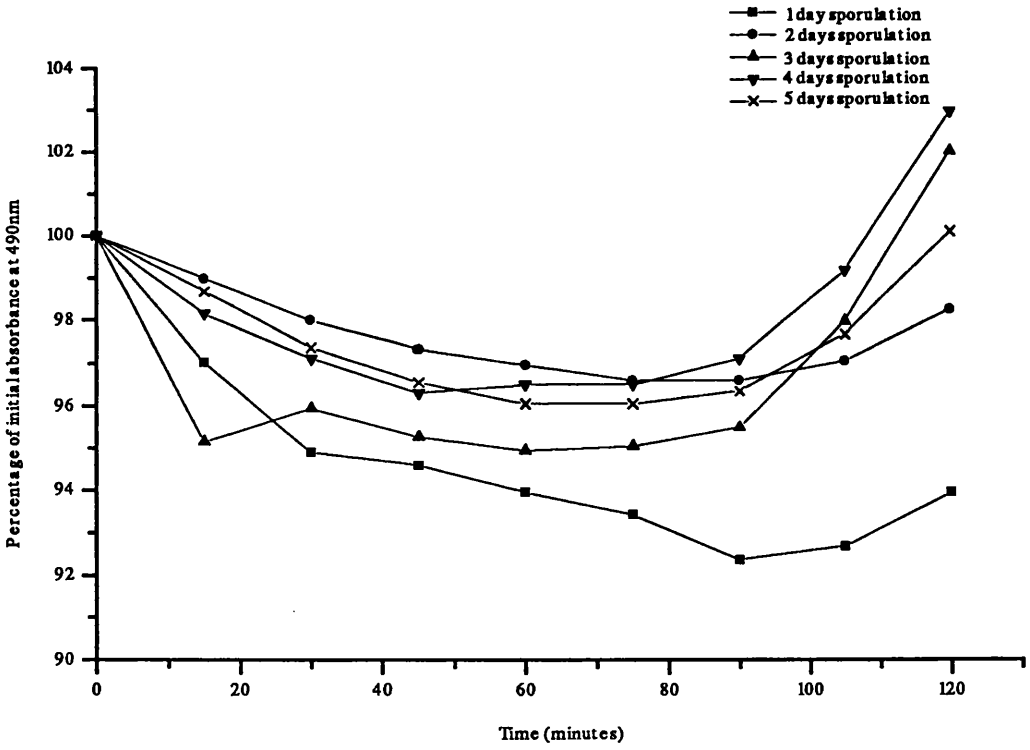
There is great interest in factors that can be exploited to increase the initial rate of germination of spores, without having deleterious effects on other spore characteristics, such as heat resistance. The increase in germination rate could be useful to reduce the readout time for the acid indication of growth used in the Attest. A more rapid activation

of alpha-glucosidase, causing a more rapid result for the fluorescence assay, may also be a result of increased germination of spores.

4.3.3.1 Effect of length of sporulation

B.stearothermophilus spores were produced using standard media. One batch was harvested after each of 1, 2, 3, 4 and 5 days.

Figure 4.4. The effect of the length of sporulation on the germination of *B.stearothermophilus* spores, ATCC 7953, in TSB at 60°C



The spore crops were cleaned and stored at 4°C. The germination was measured at 60°C at 1 hour intervals for 8 hours (Figure 4.4). The germination profiles for the spores harvested between 2 and 5 days were very similar. However, the spores that had been produced in 24 hours had a greater initial rate of germination over the first 30 minutes, and germinated to a greater extent in 2 hours.

This could be useful in producing spores more rapidly that would have an improved germination rate compared to the standard spores (sporulation time, 3 days). However, effects of a short sporulation time on other spore characteristics must be investigated.

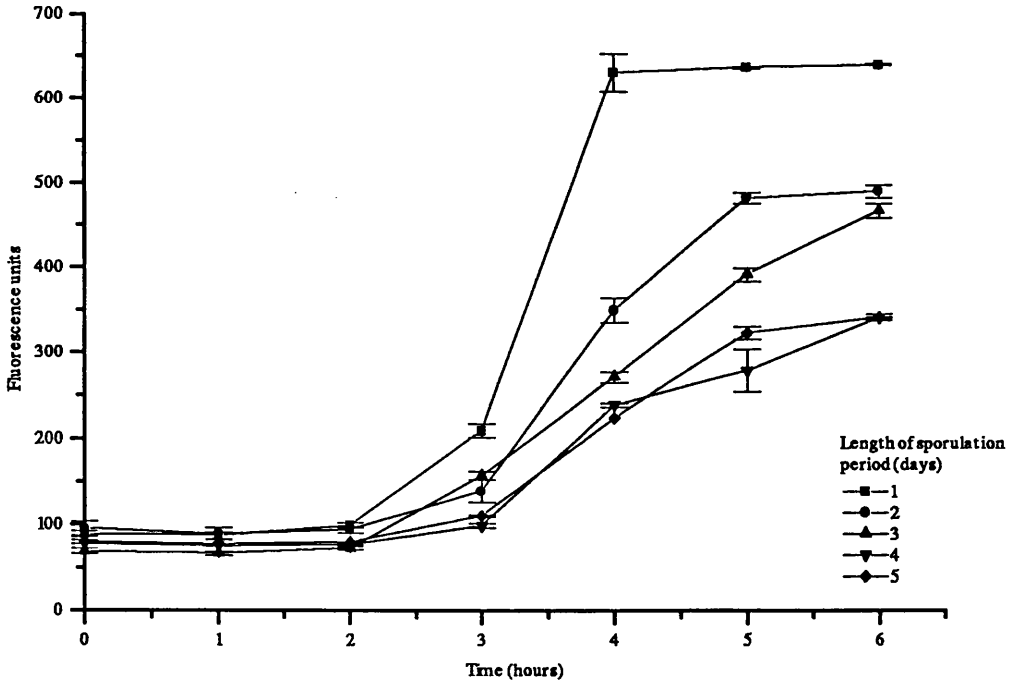
4.3.3.2 Effect of sporulation time on the enzyme activity of spore suspensions during germination

One hundred microlitres of each of the 1, 2, 3, 4 and 5 day spore batches (4.3.3.1) of *B.stearothermophilus*, ATCC 7953 were used to inoculate 75ml of TSB in a 250ml conical flask. The culture was incubated at 60°C in a shaking incubator. Three replicate 100µl samples were taken at hourly intervals over a 6 hour period. Fifty microlitres of 4-MUG solution (0.1g/l) and 50µl of phosphate buffer pH 7.4 were added to each sample in a 96-well microtitre plate. The fluorescence of the samples was determined (*3.2.2*).

Figure 4.5 shows the effect of the length of sporulation on the activity of alpha-glucosidase during the germination of the spores. There was no increase in alpha-glucosidase activity above the basal level during the first 2 hours of incubation in any of the spore crops. After this period, the spores started to produce increasing amounts of enzyme. The highest levels of alpha-glucosidase were detected during germination of the spores that were produced in 1 day. The activity of alpha-glucosidase decreased as the length of sporulation of the spore crops increased. The lowest activity was detected in

spores that had been produced in 5 days. Since the levels of enzyme remained the same over the first 2 hours of germination at 60°C, this reveals that the enzyme measured in the Attest Rapid Readout Biological Indicator is not produced during germination, but is merely present in the extracellular medium of the spore suspension prior to being dried onto the spore strip.

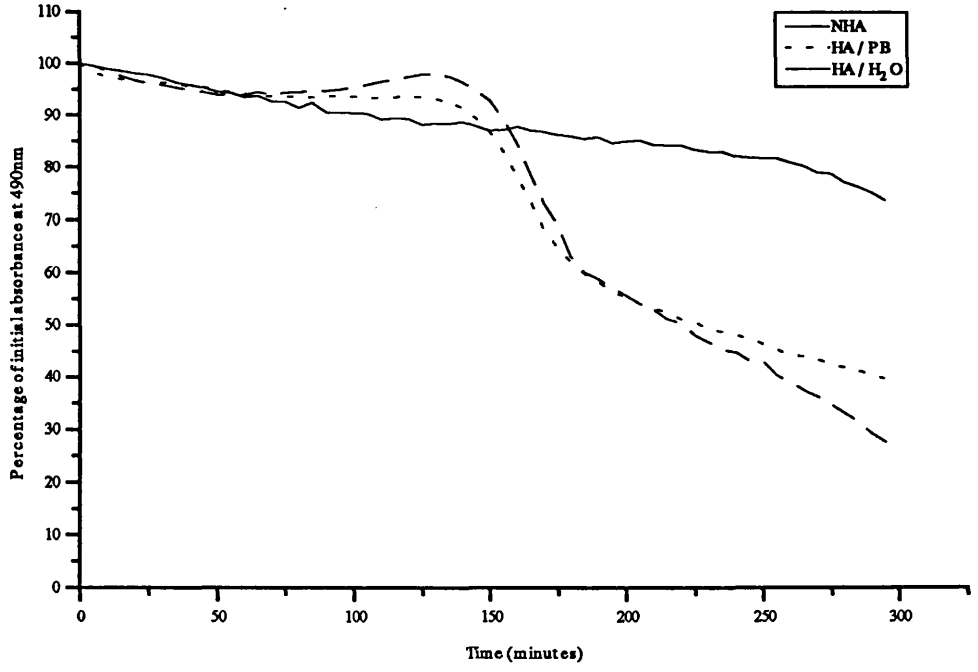
Figure 4.5. Effect of length of sporulation period on the alpha-glucosidase activity of spore suspensions during germination in TSB at 60°C



4.3.3.3 Effect of heat activation

It has been reported that treatment of spores with sub-lethal heat has the effect of increasing the viable count of a spore population. Sub-lethal heat treatment is also used to destroy any contaminating vegetative cells present in spore suspensions.

Figure 4.6. The effect of heat activation on the germination of *B.stearothermophilus* spores, ATCC 7953, in TSB at 60°C, measured by absorbance at 490nm. NHA, non-heat activated spores. HA / PB, heat activated spores (10 minutes at 100°C) in phosphate buffer pH 7.4. HA / H_2O, heat activated spores (10 minutes at 100°C) in distilled water.



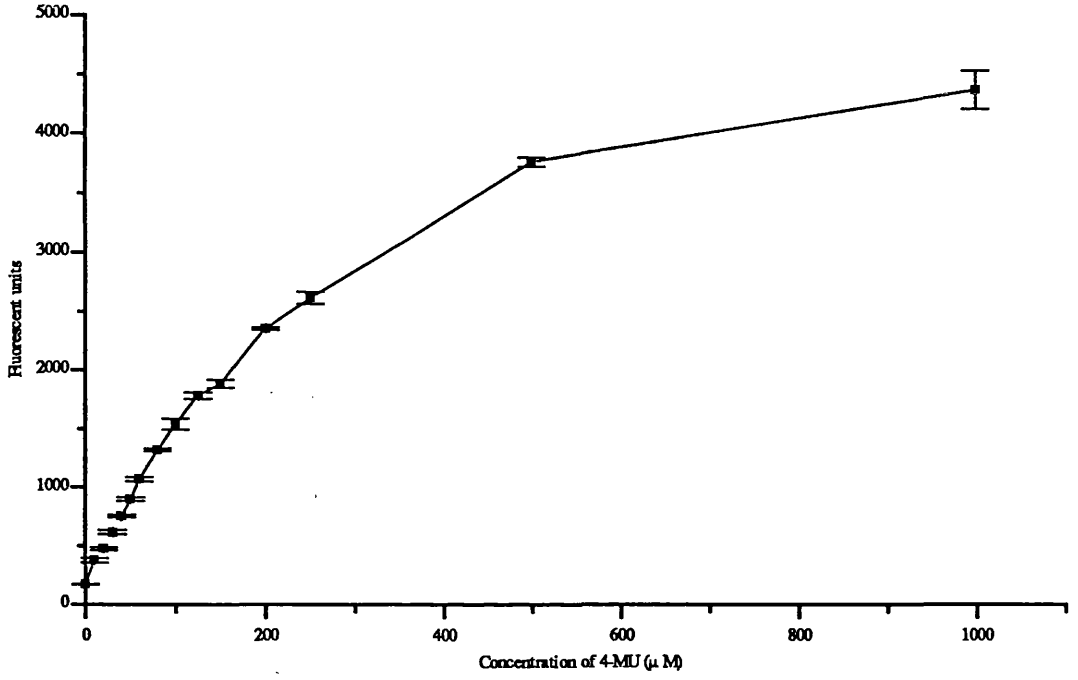
An appropriate volume of spore suspension (sporulation time, 72 hours) was diluted in either distilled water or phosphate buffer pH 7.4, to give a total count of approximately 1 x 10^8 /ml. The spore suspension was heated at 100°C for 10 minutes, then cooled in an ice water bath. The germination of the spore suspensions was determined (4.2.2) in TSB at 60°C. The effect of heat activation was to increase the rate of germination of spores, following a short period of slow germination. There was a more marked effect when distilled water rather than phosphate buffer was used as the heating medium (Figure 4.6).

4.3.4 Effect of carbohydrates on enzyme activity in spores and vegetative cells

4.3.4.1 Relationship between 4-methylumbelliferone (4-MU) concentration and fluorescence

A 1mM stock solution of 4-methylumbelliferone (7-hydroxy 4-methylcoumarin) (17.62mg / 100ml) was prepared in BM. A range of concentrations of 4-MU, between 0 and 1000µM, were produced by dilution of the 1mM stock solution in BM. The fluorescence of five replicates of each concentration was determined using a Titertek Fluoroskan II plate reading fluorimeter (Figure 4.7). The fluorescence produced is directly proportional to the concentration of 4-MU up to about 150µM 4-MU, or approximately 1500 fluorescence units. Therefore, below this concentration, the measured fluorescence can be used as a direct measure of alpha-glucosidase activity (*ie.* amount of 4-MU released by the action of the enzyme).

Figure 4.7. Calibration curve of 4-methylumbelliferone (4-MU) in basic medium (BM)



4.3.4.2 Effect of methyl-α-D-glucoside and maltose on extracellular alpha-glucosidase in vegetative cell cultures

Seventy five millilitre aliquots of BM with various concentration of methyl-α-D-glucoside (0.1%, .25%, .5%, 1.0%, and 2.0%) or maltose (0.5%, 1%, 2% and 5%) were dispensed into 250ml conical flasks and autoclaved for 15 minutes at 121°C. Seventy five millilitres of TSB in a 250ml conical flask was inoculated with frozen stock vegetative cell culture. The culture was incubated in a stirring incubator at 60°C for 16 hours. One millilitre of this culture was used to inoculate each of the flasks of media. The cultures were

incubated in a shaking incubator at 60°C for 6 hours. Three replicate 100µl samples were taken from each flask and were added to 100µl of 4-MUG solution (0.1g/l) in a 96-well microtitre plate.The fluorescence of the samples was determined.

Figure 4.8. Effect of maltose and methyl-α-D-glucoside concentration on α-glucosidase in vegetative cell cultures in BM, measured by the fluorescent substrate, 4-MUG

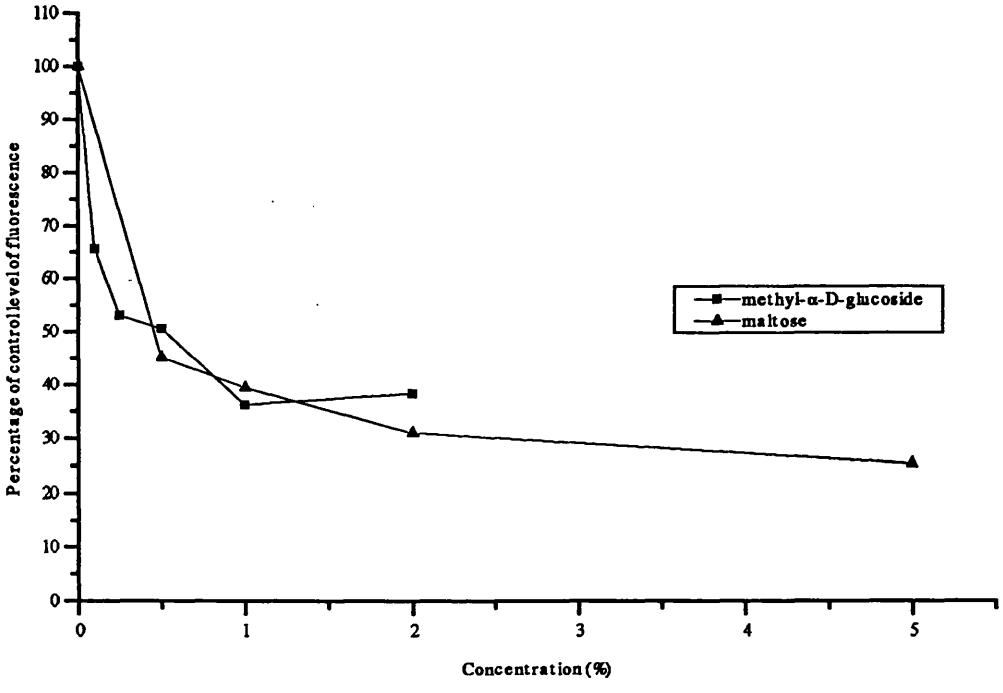


Figure 4.8 shows the relationship between the concentration of methyl-α-D-glucoside or maltose in the culture medium and the alpha-glucosidase level in 6 hour (logarithmic phase) cultures. The highest extracellular alpha-glucosidase level was found in the

unsupplemented cultures. Increasing the concentration of methyl-α-D-glucoside or maltose caused inhibition of extracellular alpha-glucosidase in the vegetative cell cultures. Maltose and methyl-α-D-glucoside exerted either an inhibitory effect on the production and / or the expression of alpha-glucosidase during vegetative cell growth.

4.3.4.3 Effect of various carbohydrates on the activity of alpha-glucosidase in spore suspension and crude extracts

The effect of various carbohydrates on the α-glucosidase activity in crude spore extracts or whole spore suspensions was determined in phosphate buffer pH 7.4, using the 4-MUG assay. Enhancement of the enzyme activity by the presence of a particular carbohydrate in buffer solution would indicate further studies to elucidate its action on α-glucosidase activity in the BI recovery medium.

A stock solution of 10mM concentration of each of the following carbohydrates was prepared: glucose, maltose, methyl-α-D-glucoside, maltotriose, maltotetraose, maltopentaose, maltohexaose, trehalose, nigerose and isomaltose. Appropriate volumes of the stock solutions were pipetted into microtitre plate wells to give a range of concentrations of each sugar up to 8mM.

Fifty microlitres of whole spore supension (or 10µl of crude spore extract) were added, with 20µl of 4-MUG solution (0.1g/l), and the volume made up to 300µl with phosphate buffer pH 7.4. The fluorescence of the samples was determined. Figure 4.10 shows the effect of a range of concentrations of carbohydrates on the activity of alpha-glucosidase.

Figure 4.10. Effect of various carbohydrates on the alpha-glucosidase activity in crude spore extract, ATCC 7953, measured by the 4-MUG assay

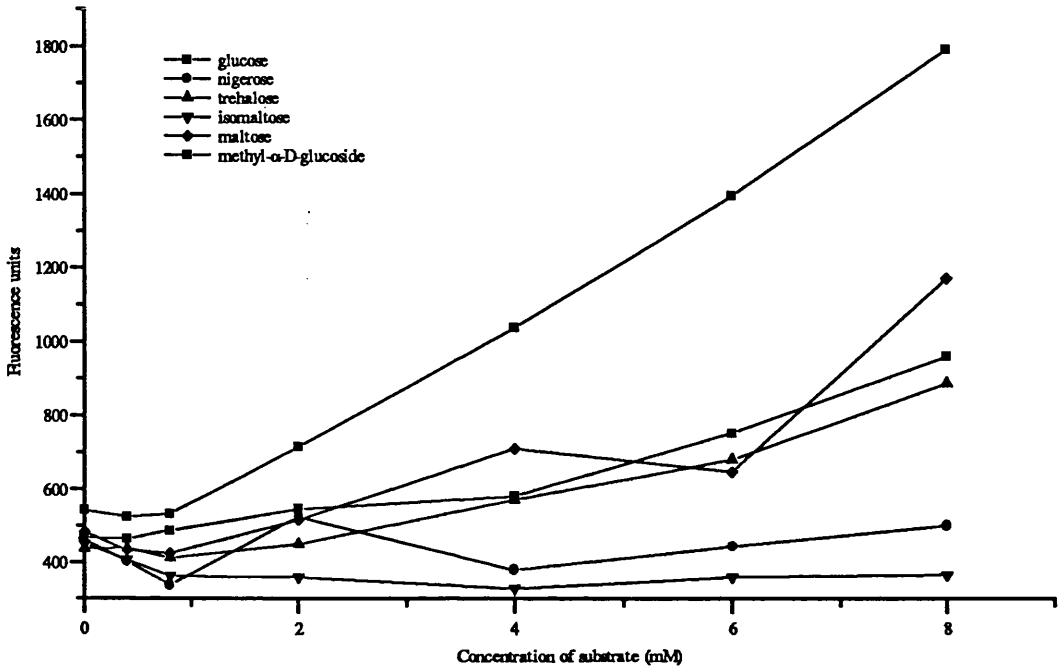
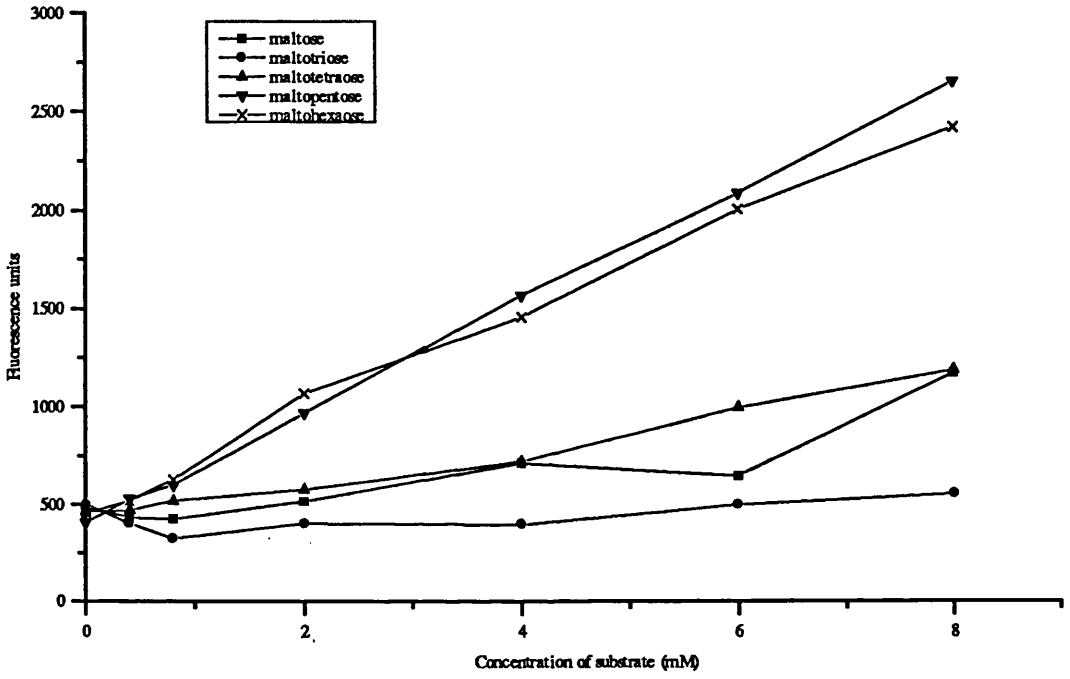


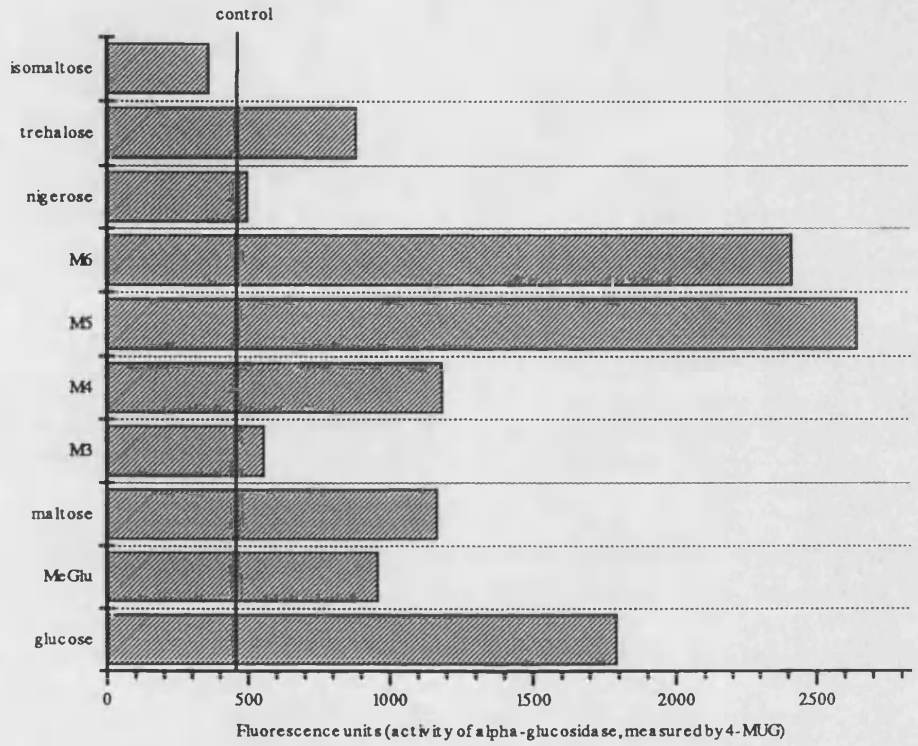
Figure 4.11 shows the effect of increasing the number of residues in the malto-oligosaccharide series. This leads to an enhancement of alpha-glucosidase activity. Maltopentaose and maltohexaose follow an approximately linear increase in enhancement of alpha-glucosidase activity from 0 to 8mM concentration. These sugars give a five-fold increase in the enzyme activity compared to the control level. Maltose and maltotetraose appear to cause a small increase in activity, whilst maltotriose has apparently no effect.

Figure 4.11. Effect of number of residues in malto-oligosaccharides on the alpha-glucosidase activity in crude spore extract, measured by the 4-MUG assay



A comparison was made of the results of the response of the alpha-glucosidase enzyme to 8mM concentrations of each of the carbohydrates (Figure 4.12).

Figure 4.12. Relative effect of substrate on the activity of alpha-glucosidase, measured by the 4-MUG assay. MeGlu, methyl-α-D-glucoside. M3, maltotriose. M4, maltotetraose. M5, maltopentaose. M6, maltohexaose.



4.3.5 Effect of different carbohydrates on the growth and production of α - glucosidase in vegetative cell culture

Basic medium (BM) was prepared and divided into 75ml aliquots in 250ml conical flasks. The media were supplemented with the carbon sources shown in Table 4.1. The media were autoclaved at 121°C for 15 minutes. The flasks were inoculated with an overnight culture of *B.stearothermophilus* ATCC 7953 grown at 60°C in TSB. The cultures were

incubated in a shaking incubator at 60°C. Samples were taken after 6 hours incubation, and the viable count of the culture was determined. The alpha-glucosidase activity was determined using 4-MUG substrate in phosphate buffer pH 7.4 (*3.2.2*).

Table 4.1. Effect of carbon source on the growth and alpha-glucosidase production during vegetative growth

Supplemental carbon source	*Alpha-glucosidase activity (relative fluorescence)*	*Viable count / ml*	*Specific activity relative fluorescence per 10^4 cells*
none	3262	3.2×10^7	0.1019
lactose			
0.2%	3001	8.05×10^6	0.3728
0.5%	2984	4.95×10^7	0.0603
1.0%	2678	1.55×10^7	0.1728
glucose			
0.2%	2021	1.24×10^8	0.0163
0.5%	1050	7.00×10^4	150.0
1.0%	589	4.5×10^4	130.9
sucrose 0.5%	511	2.5×10^4	204.4
galactose 0.5%	2780	1.23×10^8	0.2260
mannose 0.5%	2004	6.55×10^7	0.3060
xylose 0.5%	1849	5.5×10^7	0.3362
glycerol			
0.2%	2549	1.15×10^6	22.17
0.5%	2496	8.05×10^7	0.3101
1.0%	1910	9.21×10^7	0.2074
soluble starch			
0.2%	2677	1.86×10^8	0.1439
0.5%	1337	4.05×10^7	0.3301
1.0%	1353	9.00×10^4	150.33

4.3.6 Study of media for the increase in fluorescence and indication of acid production during germination and outgrowth of spores

Following increased fluorescence found with various sugars when tested in phosphate buffer pH 7.4, it was decided to investigate the possibility of combining the use of various sugars in growth media to improve the rate and extent of production of the fluorescent product, without losing the ability of the spores to produce sufficient acid to affect the pH -sensitive dye, bromocresol purple.

Maltopentaose caused the highest enhancement of alpha-glucosidase of all the sugars tested in the phosphate buffer pH 7.4 system. Glucose also had a significant effect. Therefore it was decided to investigate the effect of these sugars in the recovery medium, RM (*4.2.1*) and in CDM. A spore strip was aseptically transferred to a sterile Eppendorf tube (1.5ml). Five hundred microlitres of each of the media were added, and the tube shaken to ensure complete wetting of the spore strip. The media were incubated at 60°C. The tubes were examined periodically for colour change, and the fluorescence of a sample from each tube was assessed after 24 hours' incubation. Table 4.1 shows the colour of the pH indicator and the fluorescence of the samples after 24 hours' incubation. The unsupplemented recovery medium gave the highest fluorescence, but it did not give a positive acid indication. This contradicts the results with the RM used in the Attest ™ Rapid Readout Biological Indicator, probably due to differences in carbohydrate contents of different batches of tryptone and peptone having varying effects on the alpha-glucosidase activity and on the general spore outgrowth and metabolism. Addition of increasing concentrations of glucose to the medium caused positive acid indication but reduced the fluorescence level.

Table 4.2. Acid indication of growth and alpha-glucosidase activity, measured by the 4-MUG assay, of spore strips incubated in various recovery media at 60°C

Recovery Medium	*Colour of indicator after 24 hours*	*Fluorescence units after 24 hours*
RM	P	940
RM + M5	Y	175
RM + 100mM glucose	Y	107
RM + 50mM glucose	Y	122
RM + 25mM glucose	Y	125
RM + 12.5mM glucose	Y	144
RM + 6.25mM glucose	Y	572
CDM + 100mM glucose	P	0
CDM + 50mM glucose	P	0
CDM + 25mM glucose	P	0
CDM + 12.5mM glucose	P	0
CDM + 6.25mM glucose	P	0

The spores were not capable of sufficient outgrowth and metabolism in the CDM to produce positive acid indication after 24 hours. Prolonged incubation for up to 5 days did not alter the results of any of the assays. These media tests revealed a dilemma of the pH of the medium being sufficiently low to give a positive acid indication (pH 5.3-6.8), but sufficient activity of the enzyme at that pH, and also sufficient absolute fluorescence at low pH values.

4.4 Discussion

The performance of the Attest ™ Rapid Readout Biological Indicator depends on the detection of active alpha-glucosidase following sterilisation at 132°C. Following exposure to the sterilisation process, the BIs are incubated for 1 hour at 60°C before reading the result of the fluorescence assay. Initial strategies to modify the performance of the biological indicator focussed on altering the germination response of the spores by modifying the spores themselves, or by varying the external germinating conditions.

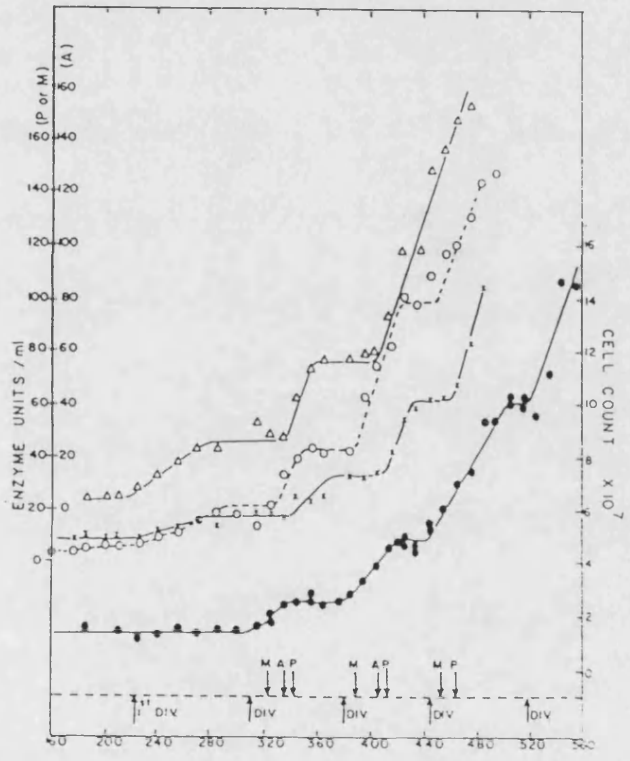
From trials of production of spores, it was found that the addition of 0.5% maltose to the inoculum and sporulation medium led to an increase in the amount of α-glucosidase per spore. However, the yield of cells in the inoculum and the spore crops were reduced by the addition of maltose. Increasing the amount of maltose beyond 0.5% led to a reduction in the level of the enzyme. Addition of maltose to the vegetative cell culture alone led to an overall reduction in the level of the enzyme, due to a reduction of growth of the culture. When these "maltose-induced" spores were allowed to germinate in TSB at 60°C, they showed a three-fold increase in the initial rate of germination compared to spores produced in standard conditions. A similar germination profile of the two spore batches was observed in CDM. The effect of glucose in the CDM on the two spore crops was studied. In the case of both groups of spores, germination proceeded more rapidly and to a greater extent with no added glucose. The "maltose-induced" spores, which had increased α-glucosidase concentration, germinated more rapidly than standard spores, both in CDM with and without added glucose (7.5mM).

This concentration of glucose is a known inhibitor of α-glucosidase [124]. It is possible, therefore, that the improved germination properties of "maltose-induced" spores are a result of increased production of α-glucosidase. Furthermore, the

inhibition of germination of standard and "maltose-induced" spores could be due to the inhibition of the enzyme activity by glucose. A potential role of α-glucosidase in germination would be more likely to involve the later stages, rather than an initial effect, supplying energy to the outgrowing cell. However, the addition of maltose to the sporulation medium has also had the effect of altering the initial stage of the germination response during the first few minutes. Maltose-induced spores were assembled into Attest units, and their performance compared to that of standard spores. No significant change in the performance of the biological indicator was found, even though there was an increase in spore-associated α-glucosidase, as demonstrated by the increase in the spore α-glucosidase (92 700 Da) band on the SDS-PAGE, with no increase in the vegetative cell enzyme band (86 700 Da), and increased levels in spore extracts demonstrated by PNPG assay.

Other workers have studied the synthesis of enzymes during spore outgrowth. Synthesis of three enzymes, α-glucosidase, L-alanine dehydrogenase and alkaline phosphatase, was studied during outgrowth of *B.cereus* strain T spores [162]. Their synthesis began at a specific time following germination, and doubled only during a fraction of the generation time (Figure 4.14). RNA and protein synthesis occur a few minutes after germination begins, but DNA synthesis does not occur until 80-120 minutes later [162]. The dormant spore is essentially lacking in stable, functional mRNA, and the conversion to vegetative growth is dependent on new mRNA transcription. During outgrowth of spores, the first few cell divisions are synchronous, the first signs of cell division occuring after 220 minutes in heat-activated spores of *B.cereus* strain T spores in synthetic medium at 25°C. The pattern of synthesis of each of the enzymes follows the same pattern for several cycles of division, after which the synchrony of division is lost. It appears that following outgrowth, an orderly process of sequential transcription and translation is operative.

Figure 4.14. Timing of enzyme synthesis during synchronous growth of *Bacillus cereus* strain T spores [162]. Spores were germinated in synthetic medium at 25°C. Samples were assayed for α-glucosidase (M) (o), alkaline phosphatase (P) (x), and L-alanine dehydrogenase (A) (Δ). Cell counts (•). In the lower bar graph, the time of initiation of cell division (DIV) and of enzyme synthesis (M, A, P) are indicated by arrows.



Spores produced over varying sporulation periods were found to have differing germination properties. There was a distinct difference in the germination profile of spores produced in 1 day compared to those produced over 2 days or longer. Those spores produced in 2 days or longer had an essentially similar germination profile. It is possible that spores harvested after 1 day are not fully mature. It is likely that the spore coats are fully functional since incomplete spore coats tend to delay and reduce the germination. A maturation process seems to take place between day 1 and 2 of

sporulation, since the germination profiles of the spores produced over longer periods are essentially similar.

Initially it was thought that spores produced over 1 day might be beneficial in the production of BIs. Higher turnover of spore batches would be more cost-effective and the increased germination rate might prove beneficial, if related to more rapid production of alpha-glucosidase. However, there are potential problems of reduced yield of spores after only 1 day sporulation compared to longer time periods, and possible reduced heat resistance properties of such spores. Heat resistance of spores may be improved by incubation of spores at 60°C on the surface of agar plates due to a drying effect, and this may also explain the increased dormancy of spores produced over longer periods on solid media [64].

Thermophilic bacterial spores are usually produced on the surface of solid agar medium, for incubation periods between several days and two weeks. Liquid media have been unable to support sufficient sporulation, and unable to produce thermophilic spores with high enough heat resistance to function as biological indicators, compared to those produced on solid media [163]. Donnellan *et al* [164] devised a chemically-defined synthetic medium for growth, sporulation and germination of *B.subtilis*. However, modification of this medium for successful sporulation of thermophiles has not been achieved. Problems of variation between batches of medium constituents may cause inconsistencies in spore properties, including heat resistance, growth indices and germination properties.

The length of the sporulation period was studied in relation to alpha-glucosidase activity during germination of spores. Although there was no increase in the level of enzyme from the basal level during the first hour of germination of spores produced in 1 -5 days (and the basal alpha-glucosidase for the different spores was very similar), after 2 hours incubation at 60°C, there was a difference between the enzyme activity

of the various spore crops. Activity of alpha-glucosidase increased in direct relation to the length of sporulation. Spores produced in 1 day had almost twice the activity after 4-6 hours of germination than spores produced over 5 days. Inactivation of alpha-glucosidase loosely attached to the spore coats may occur following prolonged incubation in dry conditions at 60°C. However, as discussed previously, this enhanced enzyme activity in 1 day spores does not seem to be of practical significance in the function of the BI.

During experiments to study the alpha-glucosidase levels in spores germinating at 60°C, there was no detectable increase in the alpha-glucosidase activity during the first 1 hour of germination, although the reduction in optical density, as a measure of germination, was 15% of the initial value after this period of incubation. An increase in extracellular alpha-glucosidase was observed following 2 hours of incubation at 60°C. These experiments were carried out with wet spores, rather than the dried spore strips that are utilised in the BI itself. It is therefore assumed that the enzyme activity detected by the fluorescence assay following 1 hour of incubation comes from two possible sources. It may be found free in the suspending medium of the spore suspension prior to drying onto the filter paper strip and becomes solubilised on immediate wetting of the spore strips with the recovery medium. The other possible explanation is that loosely bound enzyme in the spore coats diffuses into the recovery medium during the 1 hour incubation period.

Heat activation has been shown to induce several changes in bacterial spores, including increased germination rate, changes in germination requirements, and changes in spore morphology. It is thought to increase the germination rate by increasing the permeability of the pericortex membrane, inside the inner spore coats [100, 101]. Srivastava and Fitz-James [165] reported that heat activation of *Bacillus cereus* spores resulted in a change of pattern of coat proteins on SDS-PAGE gels and an increase in the carbohydrate and free SH groups of coat proteins. Spores released

about 10% of their coat proteins into the suspending buffer [165]. The total protein and alpha-glucosidase activity in the suspending buffer following heat activation of *B.stearothermophilus* spores at 100°C for 10 minutes was studied. Extracellular alpha-glucosidase activity was reduced to 2% of its activity in non-heated spores, and no significant increase in total protein was found in the suspension medium. There was no apparent alteration in the components of extracts of non-heat activated and heat-activated spores. The difference between these results and those found with *B.cereus* spores could be due to the higher temperature required for activation of *B.stearothermophilus*, lower temperatures (causing heat activation in *B.cereus*) causing heat-induced dormancy [64].

Increased glycosylation of coat proteins was also observed after heat activation [165]. Attachment of oligosaccharides to proteins leads to a similar inflexibility of proteins as seen with disulphide bonding. Carbohydrate stabilisation of coat proteins may maintain the conformation of these proteins during heat activation, that was previously conferred by disulphide bridges that have been disrupted by the heating process. Carbohydrate attachment to coat proteins may also alter the permeability characteristics or activity of certain coat proteins important in germination.

Methods used to improve the sensitivity of the fluorescence assay of the BI must take into account the fact that a dual indicator system is in operation. The indicator used to show acid production by outgrowing spores, bromocresol purple, undergoes colour change between pH 5.2 and pH 6.8, from purple to yellow. A positive result of the growth indication occurs when the recovery medium has turned yellow in 24-48 hours. The addition of various sugars to the recovery medium had the effect of increasing the alpha-glucosidase measured by the 4-MUG assay. However, this led to insufficient acid production by the outgrowing spores. The converse situation is that factors improving acid production sufficiently to give a colour change of the bromocresol purple indicator, do not enable increased fluorescence. This is due to the

inherent low fluorescence of 4-MU at low pH values. The fluorescent properties reside in the anionic form of the 4-MU, whose pK is 7.8. Therefore at pH 5.8, approximately 99% of the 4-MU is in the unionised form, and the inherent fluorescence is very low. Even if substantial increase in enzyme activity were achieved, an increase in the measured fluorescence at low pH would not be the result. The chemically-defined medium was not suitable for the germination of the spores, even with addition of up to 100mM glucose. No detection of α-glucosidase was possible.

Differences between the readout results of trials carried out using a range of recovery media for the spore strips, and the results of Attest ™ Rapid Readout Biological Indicator, is most likely to be caused by differences in carbohydrate concentrations in the tryptones and peptones available for use in the recovery media. There are possible batch to batch and manufacturer to manufacturer variations in the levels of carbohydrate in such complex media components. Such differences will have a profound effect on the enzyme levels and germination characteristics of the spores produced. Tryptone, which is present in the basic medium (BM), is a good nitrogen source, but contains no carbohydrate. Sporulation in tryptone medium is probably induced by carbon depletion. Lower levels of tryptone in the presence of yeast extract improved the yield of spores. Yeast extract contains 16.6% carbohydrate. It is assumed that there are a variety of complex carbohydrates present that support growth without inhibiting sporulation, as glucose does. A concentration of 74μM glucose improved sporulation, but five to ten times this level drastically inhibited sporulation. The low concentration of glucose in the sporulation medium was probably rapidly metabolised by the growing cells, and its depletion then caused sporulation. The larger amounts of glucose were not used up before other nutrients became depleted, preventing sporulation. Phytone peptone, which was present in the recovery medium, contained carbohydrates, whereas other peptones commonly used contained much lower levels or no carbohydrates.

The amount of α-glucosidase produced by *B.stearothermophilus* ATCC 7953 increased approximately in parallel with the increase in cell mass, and levelled off at the onset of stationary phase. This was the same pattern of production of the extracellular α-amylase studied by Welker and Campbell [149]. The effect of some carbon sources is to increase the growth of the cell culture, but to reduce the enzyme production. Such an inverse relationship was found to exist with some of the carbon sources and the total amount of α-amylase produced by *B.stearothermophilus* [135]. Generally, the poorer the carbon source for growth, the higher the amount of α-amylase produced by *B.stearothermophilus*, *eg.* sucrose produced good cell yield, but poor enzyme production [149]. Suzuki *et al* [135] found that α-glucosidase activity was strongly induced by maltose and starch, similarly to the production of α-amylase. Cells grown on technical grade maltose or starch, however, produced unusually high levels of α-amylase. Technical grade maltose was found to contain 3.5% glucose, 0.5% maltotriose and 2.5% of higher molecular weight maltodextrins [166]. Addition of maltose, maltotriose, maltohexaose, maltopentaose and maltotetraose (10^{-4}M) to cultures in a chemically-defined medium resulted in stimulation of α-amylase synthesis by 1.2, 1.6, 1.9, 2.3 and 3 times that of the control containing sucrose. Glucose had no effect. The presence of these contaminating malto-oligosaccharides explained the induction of the α-amylase by technical grade maltose and starch. Maltose and starch themselves were found to have a much weaker effect on enzyme production.

The effect of maltose and methyl-α-D-glucoside of α-glucosidase in growing vegetative cell cultures was to reduce the overall extracellular concentration of the enzyme compared to the unsupplemented control medium. This was a dual action on the growth of the cells and to reduce the amount of enzyme produced per cell. A similar α-glucosidase from the thermophilic *Bacillus* strain KP1006 closely resembled a yeast isomaltase, which was specifically induced by methyl-α-D-glucopyranoside in *S.cerevisiae* [167]. Induction of β-glucosidase was effected by a range of compounds

in yeast, including β-methyl glucoside, but the corresponding α-compound was ineffective in induction of activity in *B.stearothermophilus* ATCC 7953 spore α-glucosidase.

Differences in the induction of different α-glucosidases can likely be explained by the wide range of substrate specificities of different enzymes which are categorised as α-glucosidases. Some enzymes have a broad substrate specificity, whereas others are only able to hydrolyse a glycosidic bond of a single conformation. The structure of the active sites will determine interaction with different substrate, inducer or inhibitor molecules.

Chapter 5

LOCALISATION OF α -GLUCOSIDASE IN BACILLUS STEAROTHERMOPHILUS *SPORES*

Chapter 5

LOCALISATION OF α *-GLUCOSIDASE IN* BACILLUS STEAROTHERMOPHILUS *SPORES*

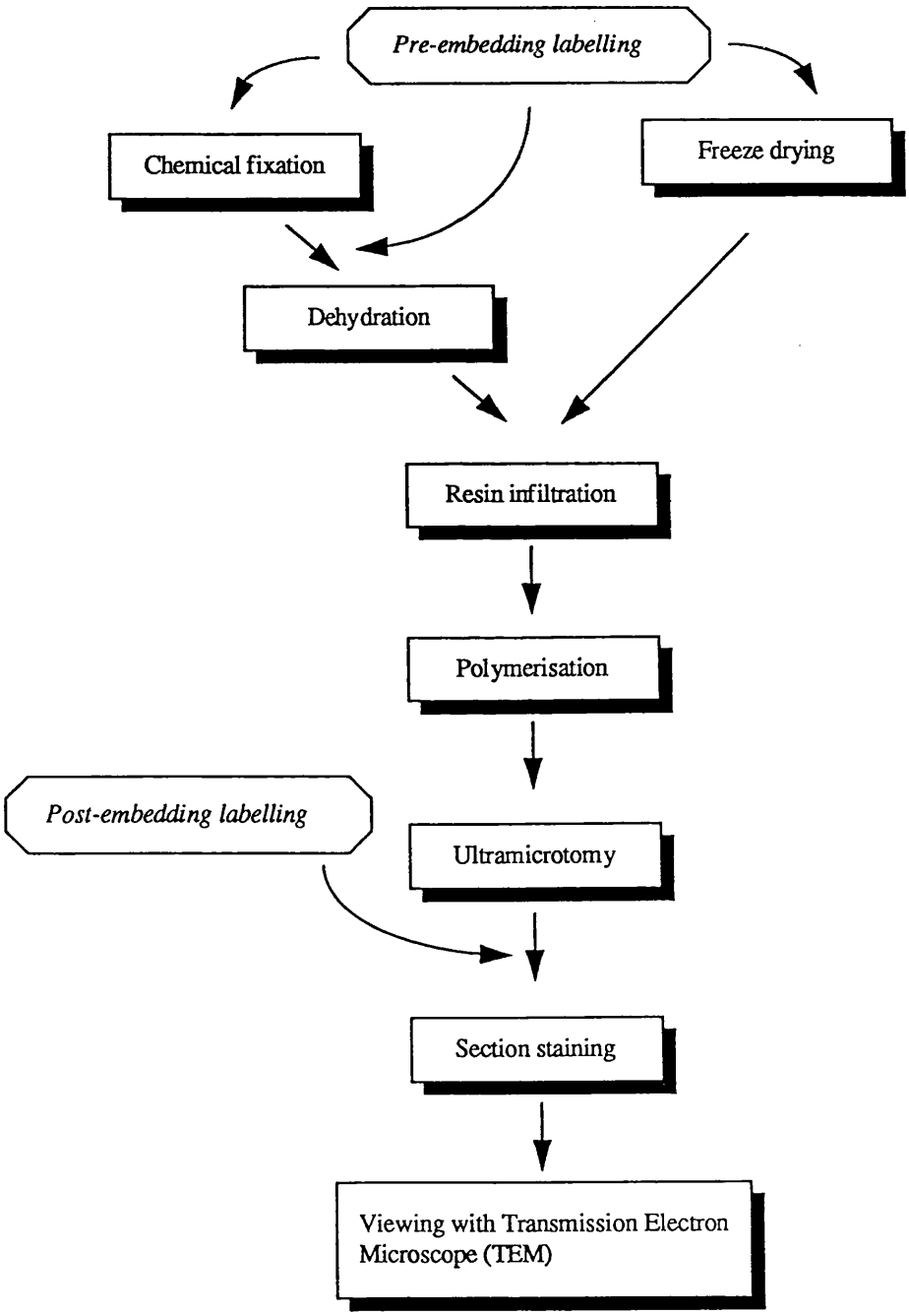
5.1 Introduction

There have been many reports of ultrastructural localisation of enzymes, and other substances, in a variety of tissues. This can be achieved either by immunological or cytochemical methods. There are many advantages to the use of immunological methods, either using monoclonal or polyclonal antibodies, providing appropriate experimental controls are used. Immunochemical methods allow greater specificity and negate many of the problems associated with cytochemical methods *ie.* permeation of chemicals into the spore, since immuno-localisation of intracellular antigens can be carried out on thin sections.

Several approaches to localisation of the α-glucosidase enzyme in spores were investigated. Pre-embedding and post-embedding localisation reactions were used. A chemical localisation method was first attempted. A more sensitive method, immunolabelling, which relies on antigenicity rather than activity, is described.

The general procedures involved in preparation of samples for pre- and post-embedding labelling (chemical or immunolocalisation) using Transmission Electron Microscopy (TEM) are shown in Figure 5.1.

Figure 5.1. Schematic diagram of processes involved in preparation of samples for immuno-labelling or chemical labelling using Transmission Electron Microscopy (TEM)



5.2 Materials and Methods

5.2.1 Preparation of spores for Scanning Electron Microscopy (SEM)

One millilitre of spore suspension was filtered through a 25mm Swinnex filter unit (Millipore Corporation, Bedford, Massachusetts) with a 0.2µm filter (Nucleopore Corporation Filtration Products, Pleasanton, California). The spores were pre-fixed by passing a solution of 2.5% glutaraldehyde and 1% acrolein (EML Electron Microscopy Laboratories Ltd. Reading, Berkshire) in 50mM phosphate buffer pH 7.4 through the filter and allowed to fix for 1 hour at room temperature. The spores were washed three times over a 15 minute period in 50mM phosphate buffer pH 7.4, by passing fresh buffer through the filter unit. A 1% solution of osmium tetroxide was prepared by diluting the 2% stock solution (Agar Scientific Ltd. Stansted, England) in phosphate buffer pH 7.4. The spores were post-fixed in 1% osmium tetroxide for 1 hour at room temperature. The spores were washed four times over a 20 minute period by passing distilled water through the filter unit. The spores were then serially dehydrated in sequentially higher concentrations of acetone, by passing the following concentrations through the filter unit, three times each over a 15 minute period; 30%, 50%, 70%, 80%, 90%, 100% and 100% dry acetone.

After dehydration the filter unit was carefully dismantled and the filters allowed to dry in a covered petri dish at room temperature. The filters were cut into small sections, or fragments of spores removed from the filters, and adhered to a 25mm diameter aluminium planchette using a 12mm diameter carbon adhesive disc (Agar Scientific Ltd. Stansted, England). The specimens were coated with gold (Edwards 150B gold sputter coater, Crawley, England) for 4 minutes, in short pulses to avoid overheating of the samples. The samples were viewed with a Jeol JM 6310 Scanning Electron Microscope (Jeol, Tokyo, Japan) or an Associated Scanning Image Device (ASID) on a Jeol 1200EX Transmission Electron Microscope (Jeol, Tokyo, Japan).

5.2.2 Preparation of spores for TEM using Transmit EM ™ resin

This method for preparation of spores for TEM was used to study the general ultrastructure and for pre-embedding localisation experiments.

A 2ml volume of spore suspension, ATCC 7953, approximate total count 10^8 / ml, was concentrated into a 0.5ml volume, and transferred to a 1ml sterile Eppendorf tube for all subsequent manipulations. For each incubation, the spores were centrifuged at 6500rpm for 2 minutes, washed and suspended in fresh solution.

Pre-fixation. A solution of 3% glutaraldehyde (prepared from a 70% glutaraldehyde solution, EML Electron Microscopy Laboraties Ltd. Reading, Berkshire.) and 1% acrolein (EML Electron Microscopy Laboraties Ltd. Reading, Berkshire.) in 50mM Sorensen's phosphate buffer was prepared. The spores were prefixed in this solution for 24 hours at room temperature.

Wash. The spores were washed twice in 50mM phosphate buffer, over a 20 minute period at room temperature.

Post-fixation. The spores were fixed in 1% osmium tetroxide (OsO_4) in 50mM phosphate buffer pH 7.4 (prepared from 2% osmium tetroxide solution, Agar Scientific Ltd. Stansted, Essex.) at room temperature for 1 hour.

Washing. The spores were washed three times over a 30 minute period at room temperature in sterile distilled water.

Dehydration. Spores were dehydrated by incubation for 20 minutes (2 x 10 minute washes in each concentration of acetone) at room temperature in increasing

concentrations of acetone in distilled water, in the following sequence; 30%, 50%, 70%, 80%, 90%, 95%, 100% and 100% dry acetone.

Resin preparation. The preparation of Transmit EM resin (Taab Laboratories Equipment Ltd. Reading. England) is shown below. The hardness of the resin produced could be varied by changing the ratio of TH1 and TH2 (increasing the ratio of TH2 : TH1 increases the hardness of the final block). The quantities given below are for a medium hardness block, which was suitable for the spore samples.

Transmit EM resin	22g
Transmit hardener TH1	45g
Transmit hardener TH2	15g
Transmit accelerator	1.6ml

The resin and hardeners were mixed thoroughly for at least 30 minutes. Accelerator (1.6ml) was added, and the activated resin was mixed for a further 30 minutes before use. The resin was stored with desiccant in a sealed container at 4°C between uses, and was warmed to room temperature (approximately 1 hour) in a sealed container before use.

Infiltration. The spores were suspended in the following series of mixtures of acetone and resin.

3:1 ratio of acetone : resin	overnight at room temperature
1:1 ratio of acetone : resin	1 hour at room temperature
1:3 ratio of acetone : resin	1 hour at room temperature

100% resin	4-5 hours at room temperature
Fresh 100% resin	overnight at room temperature
Fresh 100% resin	4-5 hours at room temperature

5.2.3 Preparation of spores for TEM using LR White™ resin

This hydrophilic resin system was used for post-embedding localisation experiments and for general ultrastructure visualisation [168].

Fixation. The fixation time was 16 hours (overnight) in paraformaldehyde or glutaraldehyde solution in phosphate buffer pH 7.4 at 4°C, unless stated otherwise. Fixative solutions were prepared by dilution of a 70% stock solution of glutaraldehyde or from the following 10% stock solution of paraformaldehyde.

A 10% paraformaldehyde (PFA) stock solution was prepared by heating water and paraformaldehyde powder to 60°C, and dropwise addition of 1M sodium hydroxide solution until dissolution was effected. This solution was then diluted to the appropriate concentration in phosphate buffer pH 7.4.

Washing. Following fixation, spores were washed twice over a 20 minute period in phosphate buffer pH 7.4.

Dehydration. The spores were then serially dehydrated for 20 minutes (2 solution changes for each concentration), in 30%, 50%, 70%, 90%, then 100% dry ethanol at 4°C

Infiltration. The spores were then infiltrated with LR White resin (London Resin Company), using the following combinations of resin and dry ethanol. If the samples were to be polymerised using UV light, it was necessary to add 0.5% w/v benzoin

methyl ether (Initiator C, Lowicryl K4M resin) to the resin prior to gradual infiltration using the resin / ethanol mixtures.

3:1	ethanol : resin	1 hour
1:1	ethanol : resin	2 hours
1:3	ethanol : resin	2 hours
100% resin		overnight
100% resin		several changes over 24 hours, then storage at 4°C prior to polymerisation

Polymerisation was either carried out by heat or UV light (**5.2.4**) depending on the experiment to be carried out with the sample.

5.2.4 Preparation of spores using the freeze drying method

Freeze drying is the removal of water by the process of sublimation from a frozen material. It has been applied to the preservation of varied biological specimens, allowing preservation of samples with a very low water content, without addition of chemicals *etc.* [169, 170]. Freeze drying is carried out by an initial rapid freezing of the sample, followed by transfer to the freeze-dryer, where water is removed from the product under vacuum. Fixation by freeze drying has the potential advantage for immunological work that antigenicity is not diminished by the use of chemical fixatives. However, maintenance of ultrastructure is a potential problem.

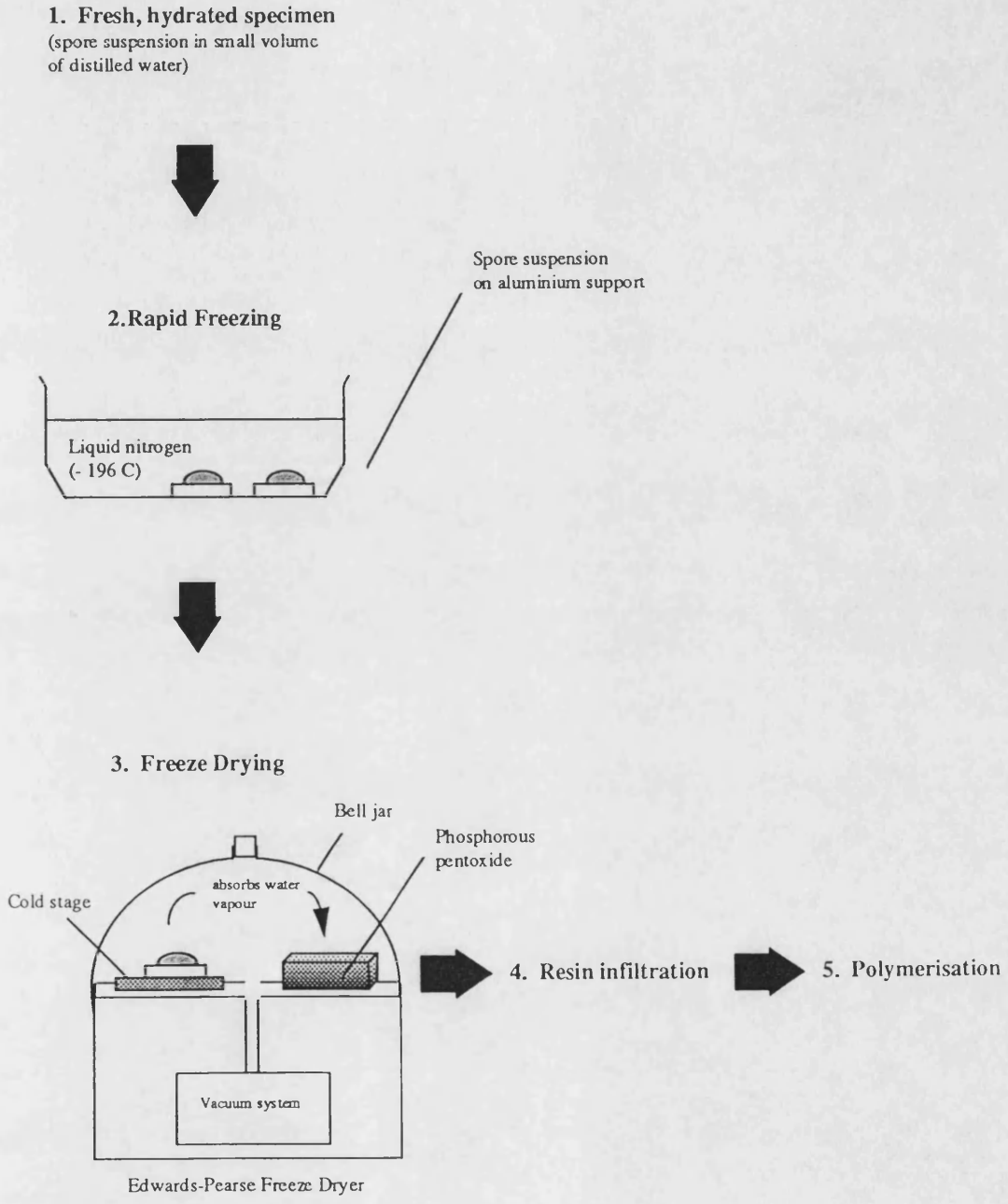
A diagrammatic summary of the freeze drying process is given in Figure.5.2. Spore samples were suspended in a minimum volume of sterile distilled water (less than 0.5ml per sample). An aluminium foil-lined polystyrene box was filled with liquid nitrogen and allowed to cool for several minutes prior to use. A metal support was placed in the liquid nitrogen, to hold the spore samples during the freezing process.

An aluminium foil container for the spores was placed on the metal support in the box. Once the aluminium container was cooled, the spore suspension was pipetted into the container. The samples were covered completely by the liquid nitrogen to ensure rapid and complete freezing to avoid ultrastructural damage to the specimen. After approximately 15 - 20 minutes, the samples were removed to the freeze dryer (Edwards-Pearse Freeze Dryer, model EPD3, Edwards High Vacuum, Manor Royal, Crawley, Sussex, England).

The aluminium containers holding the samples were transferred to the water-cooled stage in the freeze-dryer. The container in the chamber was filled with phosphorous pentoxide, which absorbed water vapour during the freeze drying process. The bell jar was placed over the chamber, and a vacuum was drawn (approximately 10^{-2} tor). The temperature guage was set to "cold", allowing the temperature in the chamber to fall to about -70°C. The samples were allowed to dry for at least 6 hours. Air was then admitted to the chamber, and the dried samples removed.

Following freeze drying, LR White resin (with addition of 0.5% benzoin methyl ether, if samples were to be polymerised using UV light) was added to the freeze dried spore suspension immediately upon removal from the freeze drier. The sample was transferred to a 1.5ml Eppendorf tube and stored for 5 days at 4°C in a desiccated environment. The resin was removed and replaced with fresh resin several times during storage. The samples were then prepared for polymerisation.

Figure 5.2. Diagrammatic summary of the freeze drying technique for treatment of bacterial spores for TEM



5.2.5 Polymerisation

5.2.5.1 Heat polymerisation

Gelatin capsules were heated at 70°C for 2 hours before use. The capsules were filled with the spores suspended in the resin. The capsules were filled completely with further resin if required. The capsules were polymerised at 18 hours at 70°C (Transmit EM resin) or 18 hours at 50°C (LR White resin) and cooled to room temperature for at least 30 minutes before sectioning.

5.2.5.2 UV polymerisation

Gelatin capsules were filled with the resin-embedded sample and extra resin was added if necessary to fill the capsules. The capsules were placed in the metal support ring in the chamber, at a distance of 20cm from the UV light source, and 20cm from the bottom of the aluminium foil-lined chamber. The lid of the chamber (aluminium foil-lined) was closed, and the UV lamp (λ_{360nm}) was switched on. The samples were allowed to polymerise for 3 days at 4°C.

5.2.6 Sectioning

The majority of biological specimens are too thick to allow an electron beam to penetrate in order to visualise the specimen with the electron microscope. For accelerating voltages up to 100kV, it is necessary for the sections to be between 10nm and 100nm [170, 171]. Sections should be of known and standard thickness, and have no cracks or wrinkles. The high resolving power of the electron microscope can be fully utilised only if high quality sections are available. Generally, semi-thin sections are cut prior to thin sectioning and stained, often with alkaline toluidine blue [172], to locate areas of interest within the specimen, and to ascertain whether fixing and infiltration have been successful. In the case of bacterial spores, insufficient detail is

available under the light microscope to achieve any benefit from semi-thin sectioning, therefore thin sections were cut directly. This section gives a description of the processes involved in production of thin sections.

5.2.6.1 Trimming and preparation of the block face

The block is mounted in the chuck. The gelatin capsule was removed from the block using a double-edged stainless steel teflon-coated razor blade (Electron Microscopy Sciences, Science Services UK Ltd., London). The top surface of the block was trimmed with a glass knife until a smooth surface was achieved, large enough to allow cutting of a face of desirable size (approximately 0.5mm on one edge). The block face could be slightly larger than this, but small block faces are desirable since less compression is produced during cutting, and leads to better quality sections. A trapezoidal shape block face was cut with the double-edged razor blade.

5.2.6.2 Preparation of glass knives

Glass knives were prepared immediately prior to use, using the LKB Knifemaker 7801A (LKB Produkter AB, Stockholm, Sweden) and strips of ultramicrotome glass (Agar Scientific Ltd., Stansted, England). Storage of glass knives lead to loss of sharpness due to flow at the cutting edge of the knife. The strip of glass was scored and cut in half to facilitate handling. The glass was washed in warm water and detergent, then dried with paper towel. The glass was then stored in a dust-free environment. The length of glass was cut into squares, which were further cut into two equal halves to produce 45° knives. The cutting was achieved by first scoring the glass then applying equal pressure to each side of the score, to produce a straight, clean fracture along the score line. The knives were examined under binocular magnification to assess the suitability of the cutting edge. It was ensured that touching of the knife edge was avoided during usage.

Plastic troughs were attached to the knives using molten dental wax. The knives were then stored in glass knife storage boxes (Taab Laboratories Equipment Ltd., Aldermaston, Hampshire) to avoid contamination by dust. Dust particles caused fine nicks in the cutting edge of the knives which render them unusable. Even a high quality glass knife edge could be used only for a few ribbons of sections before necessitating changing the knife.

5.2.6.3 Thin sectioning

The block was positioned in the chuck of the ultramicrotome (OMU3, Reichert (Leica), Austria) and clamped tightly. The knife was positioned in the knife holder, ensuring that the knife and the block were not able to collide. The knife trough (LKB truf, Leica UK Ltd., Milton Keynes) was slightly overfilled with sterile distilled water from a 20ml syringe fitted with a fresh 0.22μm filter. Water was removed from the trough with a needle and syringe to obtain an almost flat meniscus, which gives optimal wetting of the knife edge. This gave a silver reflection when viewed through the binocular microscope attached to the microtome.

The block was aligned with the knife, ensuring that the bottom and top of the block were parallel to the knife and that the block face itself was in a vertical position. The reflection of the block moving downwards past the knife should be of even thickness along the length of the knife edge.

The ultramicrotome was then switched to the automatic mode, the block being advanced towards the knife by the thermal advance system until thin sections were cut. Ideally, the thin sections formed a ribbon across the surface of the water, which facilitated collection of the sections onto the grids. The thickness of the sections could be estimated by the colour they appeared on the water in the collecting trough, as viewed through the binocular microscope (Table 5.1). The thickness of sections

can be estimated within an accuracy of 10-20nm using this method. The scale is based on measurements made by Peachey [173]

Table 5.1. Interference colour index and section thickness scale.

Colour	*Section thickness*
Grey	< 60nm
Silver	60-90nm
Gold	90-150nm
Purple	150-190nm
Blue	190-240nm

The desirable thickness of sections for TEM is approximately 100nm. Such sections appear gold on the surface of the water. Unsuitable sections were removed with an eyelash attached to a cocktail stick with dental wax.

Approximately 8-10 sections were collected onto a single grid. The sections needed to be stretched prior to picking up onto the grids, due to compression that was caused during the cutting process. This was achieved either by application of heat using a heated pen, or by the use of various vapours such as chloroform. A cotton-tipped applicator was dipped in chloroform and passed across the top of the sections, avoiding touching the water or sections themselves, allowing the sections to spread slightly.

5.2.6.4 Section collection

The sections were deposited on a grid (usually nickel, gold or copper). The grid was blotted dry from the underside, and allowed to dry completely prior to being stored in a grid box before staining and viewing.

5.2.7 Staining

The following solutions for staining were prepared.

Uranyl acetate solution

Uranyl acetate (0.6g) and 20ml of 30% ethanol (prepared with sterile distilled water) were combined. The solution was filtered and stored in an amber glass bottle in the dark for up to 3 weeks.

Reynold's lead citrate solution (modified)(Reynolds 1963)

Lead acetate (1.33g) and 1.76g of sodium citrate were combined in a 50ml volumetric flask. Thirty millilitres of sterile distilled water was added. The solution was shaken vigorously for 1 minute and allowed to stand for 30 minutes, with intermittent shaking to ensure complete conversion of lead acetate to lead citrate. Eight millilitres of 1N sodium hydroxide solution (carbonate-free) was added, and the solution diluted to 50ml with sterile distilled water. The solution was ready to use once the lead citrate had dissolved, and could be kept up to 1 month.

The grids were placed section side down on drops of boiled distilled water, supported on a piece of dental wax. The grids were blotted dry with filter paper, and then placed on drops of uranyl acetate solution for 10 minutes. The grids were blotted dry,

and then placed on drops of lead citrate solution (in a covered petri dish with filter paper soaked in 10M NaOH). The grids were placed on drops of distilled water, rinsed with running boiled distilled water, then blotted dry. The sections were then viewed using a Transmission Electron Microscope (Jeol JM 1200EX, Tokyo, Japan) (funded by Science and Engineering Research Council) at an accelerating voltage of 80kV.

5.2.8 Chemical localisation of α-glucosidase using hexazonium pararosaniline

The chemical localisation of α-glucosidase was adapted from methods to demonstrate other hydrolytic enzymes, such as cholinesterase [174] and β-glucuronidase [175]. The method involves the reaction of the enzyme with a specific substrate to release naphthol. The coupling agent (hexazonium pararosaniline [176, 177]) reacts with the naphthol in the tissue to produce an electron-dense, insoluble precipitate.

Figure 5.3. Schematic representation of the chemical localisation reaction

Alpha-glucosidase + naphthyl-α-D-glucoside → naphthol + glucose

Naphthol + coupling agent → insoluble electron dense precipitate

5.2.8.1 Preparation of hexazonium pararosaniline [176]

Solution 1. One gram of pararosaniline was dissolved in 30ml of hot 2N hydrochloric acid. The solution was cooled and filtered.

Solution 2. One gram of sodium nitrite was dissolved in 30ml of distilled water. The solution was filtered.

Equal quantities of solution1 and solution 2 were mixed in a test tube immediately prior to use. Diazotisation was completed after 1 minute at room temperature, resulting in a clear, straw-coloured solution.

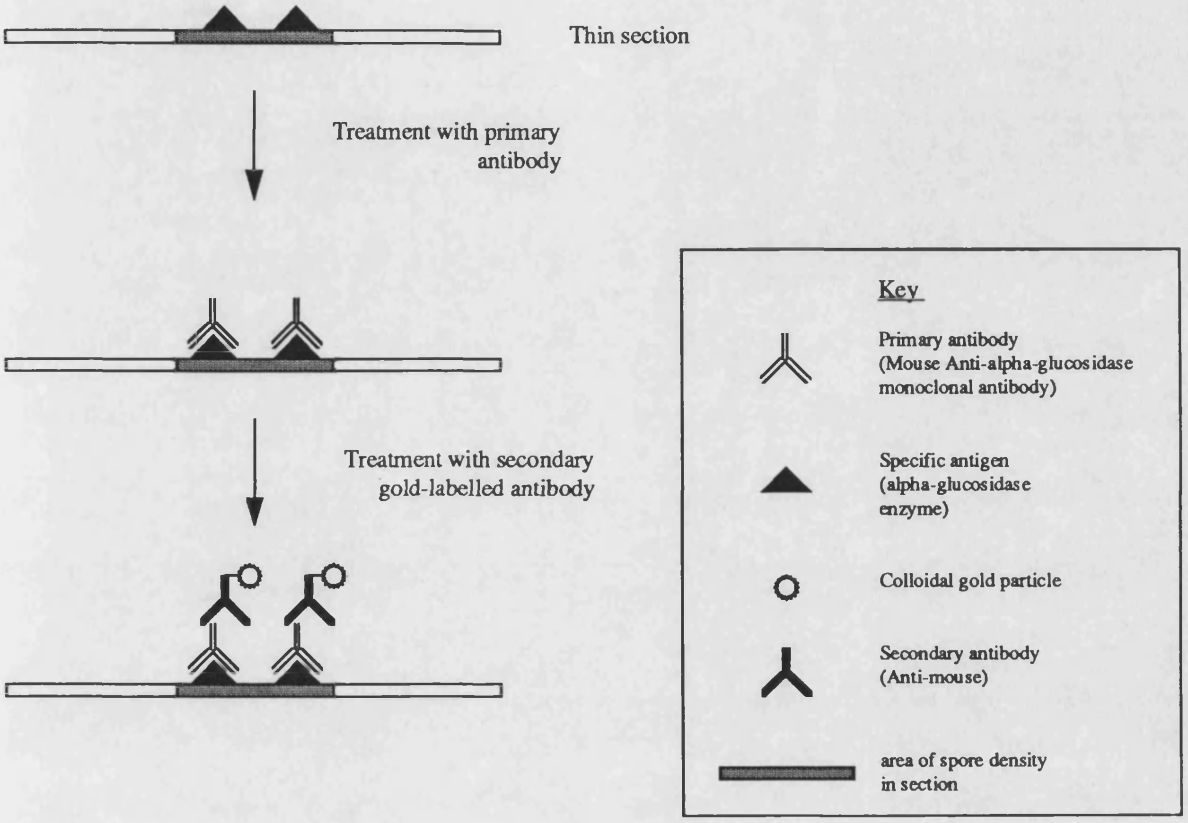
5.2.8.2 Chemical localisation reaction

A solution of 0.2mM naphthol-α-D-glucoside, 1.8mM hexazonium pararosaniline in 0.1M acetate buffer pH 5.2 was prepared. The sections were placed on drops of distilled water, then incubated in this solution in a covered microtitre plate at 60°C for 30 minutes. The grids were washed thoroughly in phosphate buffer pH 7.4 prior to storage or staining with uranyl acetate and lead citrate.

5.2.9 Immunolocalisation of α-glucosidase

A two-step indirect immunolabelling method was employed (Figure 5.4). An unconjugated mouse anti-α-glucosidase antibody (primary antibody) was allowed to bind to the α-glucosidase on the thin sections. A gold-conjugated goat anti-mouse antibody (secondary antibody) was then allowed to bind to the primary antibody to allow visualisation under TEM.

Figure 5.4. Schematic representation of the post-embedding mmunolocalisation reaction



5.2.9.1 Preparation of anti-α-glucosidase monoclonal antibody

Hybridisation between Balb/c NS-1 myeloma cells and spleen cells from immunised Balb/c mice was performed. Balb/c mice were immunised 3-5 times with *B.stearothermophilus* ATCC 7953 spores by interperitoneal injection at 2-3 week intervals, with a final injection of 1 x 10^6 cells given intravenously 3 days prior to aseptic removal of the spleen. Splenic leucocytes were combined with NS-1 myeloma cell line in 35% polyethylene glycol. The treated cells were allowed to recover for 24 hours in Heaps Dulbecco's Modified Eagle Medium (HDMEM) with 10% foetal calf serum. The cells were diluted to 7.5 x 10^5/ml in hypoxyanthene aminopterin thymidine (HAT) medium, which is selective for hybridomas, and plated in 96-well plates. The medium was replaced as required and observed for the appearance of

hybridoma growth. The viability of the hybridomas was determined every 2 days using a haemocytometer counting chamber. Supernatants from the resulting hybridoma cultures were screened for the production of spore-specific antibody. Six cell lines were chosen and cultures were produced for further study.

Alpha-glucosidase-coated particles were prepared. IDEXX carboxylated particles were added to sodium phosphate buffer pH 7.0, and allowed to incubate for 30 minutes at 37°C. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) (0.5mg/ml), α-glucosidase (1mg/ml) from *B.stearothermophilus*, and blotto blocking agent (2% nonfat dry milk in borate buffer) were added. The beads were centrifuged and the pellet suspended in 0.05% sodium azide in sodium phosphate buffer pH 7.0, and stored at 4°C prior to use.

Supernatants of the six cell lines were screened by Particle Concentration Fluorescence Immunoassay (PCFIA) using the IDEXX Screen Machine (IDEXX Corporation. Portland). Twenty microlitres of the cell culture supernatant and 20µl of α-glucosidase-coated particles were added to each well of the IDEXX plate, and incubated for 30 minutes at 22°C. The plates were washed twice in the IDEXX machine with 0.0125% NP40 (Particle Data Lab Ltd.) in PBS. Twenty microlitres of goat anti-mouse IgG-FITC (5mg/ml) was automatically added. The plates were incubated at 22°C in the dark for 30 minutes, washed in PBS-NP40, and the bound fluorescence was determined. Lysozyme-coated beads and miscellaneous cells were used as a negative control. Intact spores of *B.stearothermophilus* were used as a positive control. Two of the six cell lines which showed the highest levels of bound fluorescence were chosen and injected into pristaned mice for expansion of the monoclonal.

Balb/c mice primed with approximately 0.3ml pristane and allowed to rest for 10 days were injected intraperitoneally with approximately 2 x 10^6 cells to induce ascites. The

ascites fluid was collected and the monoclonal antibodies were purified using the Bio-Rad Laboratories Affi-Gel Protein A MAPS 11 system (Bio-Rad Laboratories, Hercules, California). The monoclonal antibody was precipitated from the ascites fluid by dropwise addition of 50% saturated ammonium sulphate. The precipitate was allowed to incubate on ice for 2 hours, centrifuged at 10 000 rpm for 20 minutes at 4°C. The pellet was suspended in PBS and dialysed overnight in PBS at 4°C with 4 changes of buffer.

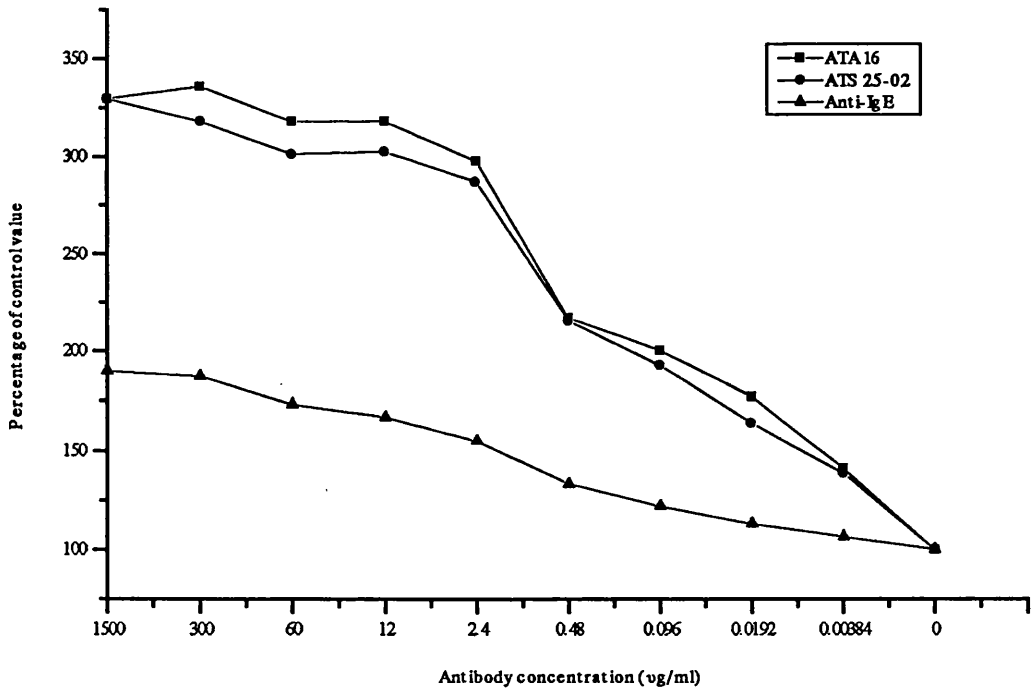
A 1½ inch Protein A column was equilibrated with 60ml of PBS at 22°C. The antibody solution was added, unbound protein was washed off the column with 60ml of PBS. The column was then eluted with the elution buffer provided with the MAPS 11 kit. The antibody was collected in 2ml fractions, and the pH adjusted to pH 7.0. The immunoglobulin fraction eluted in the first eight 2ml fractions. Samples with the highest absorbance readings (280nm) were pooled and dialysed overnight in PBS with 4 changes of buffer. The column was washed with 60ml of PBS, and regenerated with 40ml of regeneration buffer, provided with the MAPS 11 kit. The column was again washed with 60ml of 0.05% sodium azide in PBS and stored at 4°C. Sodium azide 0.02% was added to the pooled antibody, prior to its storage at 4°C.

5.2.9.2 Testing of specificity of anti-α-glucosidase monoclonal antibodies

Fifty microlitres of different dilutions of α-glucosidase from *B.stearothermophilus* (Sigma Chemical Co., St. Louis) in 0.1% BSA in PBS pH 6.8 were added to individual wells of a Dynatech microfluor plate (Dynatech Laboratories, Chantilly). Fifty microlitres of different concentrations of 4-MUG in PBS pH 6.8 was added to each well. The plate was incubated at 37°C, and the fluorescence was recorded at 5 minute intervals. The optimal enzyme concentration was 40µg/ml and the optimal substrate concentration was 16µg.ml. Addition of the monoclonal antibodies to the

assay system was carried out to investigate whether inhibition of the α-glucosidase activity was caused by antibody binding.

Figure 5.5. Effect of monoclonal antibody concentration on antibody binding and α-glucosidase activity. The monoclonal antibodies were incubated with α-glucosidase-coated beads for 30 minutes at 22°C. Bound antibody was reacted with goat anti-mouse-FITC antibody and the percentage of the control level of fluorescence was determined.



The monoclonal antibodies were diluted from 1.5mg/ml to 3.84ng/ml in 0.1% BSA in PBS pH 6.8. Fifty microlitres of 16μg/ml α-glucosidase was added to each well of the microfluor plate. Five microlitres of diluted monoclonal antibody was added, and the plate incubated for 30 minutes at 37°C.

Fifty microlitres of 4-MUG (40μg/ml) was added to each well, and the fluorescence was measured immediately, and at 5 minute intervals. An anti-IgE monoclonal antibody was diluted to the same concentration as the anti-α-glucosidase antibody, and used as a negative control. This antibody had no effect on the assay system. The monoclonal antibodies from neither ATA 16 or ATS 25-02 had any inhibitory effect on the reaction. Therefore the antibody-binding site must be distinct from the active site of the enzyme. Figure 5.5 shows that the two anti-α-glucosidase monoclonal antibodies were specific for the enzyme, but that the anti-IgE monoclonal antibody was not.

5.2.9.2 Preparation of blocking buffers

Tris (tris-hydroxymethyl-aminomethane) buffer pH 8.2

Tris	0.242g (20mM)
Sodium azide	0.13g (20mM)
Sodium chloride	0.9g (225mM)
Distilled water	100ml

The buffer components were added to the distilled water. The pH was adjusted to pH 8.2 with 0.1N hydrochloric acid or 0.1N sodium hydroxide, and the solution filtered with a 0.2μm filter. This buffer was used to prepare the blocking buffers used for immunolocalisation.

BSA / Tween 20 blocking buffers

Bovine serum albumin (BSA)	0.1 - 1%
Tween 20	0.1 - 1%
Tris buffer pH 8.2	to 100%

Gelatin blocking buffers

Gelatin	0.01 - 0.5%
BSA	0.1%
Tween 20	0.1%
Tris buffer pH 8.2	to 100%

5.2.9.3 Immunolabelling procedure

Sections for immunolocalisation were collected on gilded nickel grids (G300 HEX, 3.05mm, Gilder Grids, Grantham, Lincolnshire). The sections were initially wetted by floating on grids on drops of sterile distilled water for 5 minutes at room temperature, and blotted gently with filter paper from the reverse side. The sections were incubated on the grids by placing then section side down on drops of blocking buffer (*5.2.9.2*) at room temperature for at least 5 minutes. The grids were transferred without blotting to 25µl drops of monoclonal antibody (3M Healthcare, St. Paul, MN) appropriately diluted in blocking buffer and incubated for 2 hours at room temperature. The grids were then washed to remove unbound primary antibody, by incubating them on a series of drops of fresh blocking buffer over a 30 minute period. They were transferred to a 25µl drop of gold-conjugated secondary antibody (Super EM Grade Gold Conjugate Goat Anti-Mouse IgG. 15nm gold, British Biocell International, Cardiff), diluted 1 in 10 in blocking buffer, and incubated at room temperature for 30 minutes. The grids were rinsed in a series of drops of fresh blocking buffer, to remove unbound secondary antibody. They were then rinsed in a stream of sterile distilled water, run down the side of a pair of forceps. The sections were then blotted dry from the reverse side of the grid before storage [171].

5.3 Experimental

5.3.1 Effect of sonication on surface morphology of spores

Samples of approximately 5 x 10^7 spores (0.5ml) of *B.stearothermophilus*, ATCC 7953, of untreated spores and spores having undergone between 5 minutes and 1 hour of sonication on ice were prepared for SEM (*5.2.1*). Unsonicated spores varied in size, shape and surface morphology (Figure 5.6). Spores that had undergone 5 minutes of sonication appeared largely unaltered, although a few spores had a crinkled surface appearance (Figure 5.7). Spores that had been sonicated for up to 30 minutes had a similar appearance to spores sonicated for 5 minutes. After 1 hour sonication a large number of spores appeared unchanged with respect to their surface morphology, although some were greatly disrupted, leaving isolated coat fragments and inner cores (Figures 5.8a and 5.8b). The reaction of individual spores to a particular treatment varied enormously.

Figure 5.6. Scanning Electron Micrograph of *B.stearothermophilus* spores, ATCC 7953, untreated. x 17 000 magnification. AO1343.

Figure 5.7. Scanning Electron Micrograph of *B.stearothermophilus* spores, ATCC 7953. Five minutes sonication. x 30 000 magnification. AO1465.

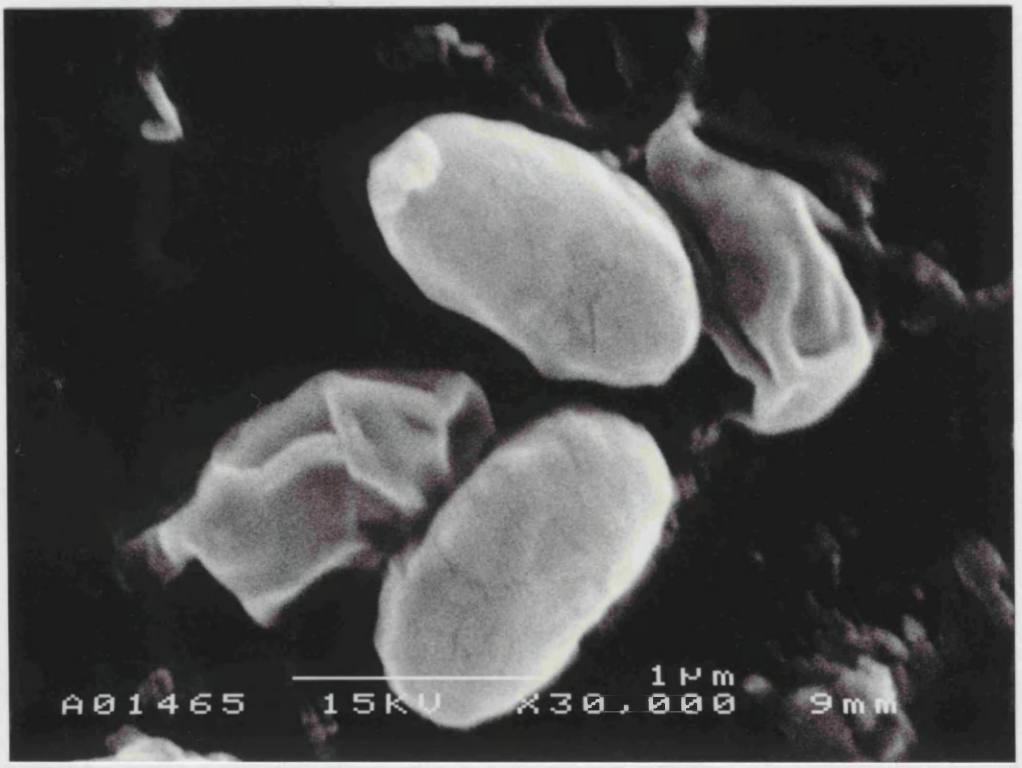


Figure 5.8a. Scanning Electron Micrograph of *B.stearothermophilus* spores, ATCC 7953. One hour sonication. x 19 000 magnification. AO1416.

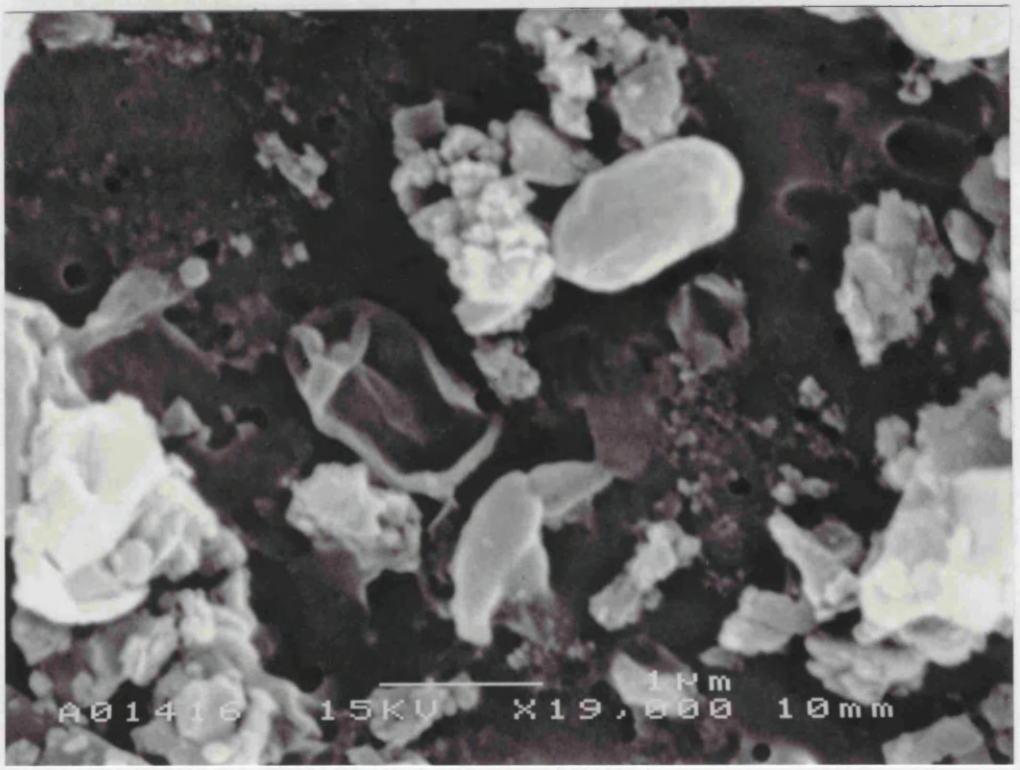


Figure 5.8b. Scanning Electron Micrograph of *B.stearothermophilus* spores, ATCC 7953. One hour sonication. x 50 000 magnification. AO1415.

The extracellular α-glucosidase activity diminished rapidly within the first hour of incubation to approximately one fifth of the original level. However, increasing the length of incubation up to 24 hours had no additional detrimental effect. Therefore a fixation time of 16 hours was chosen for preparation of spores for enzyme localisation, since the ultrastructural detail of the samples was improved, without further enzyme damage compared to shorter incubation periods.

5.3.3 Ultrastructure of spores of B.stearothermophilus, ATCC 7953

A sample of spores of *B.stearothermophilus*, ATCC 7953 were prepared for TEM using Transmit EM ™ resin (*5.2.2*) and LR White ™ resin (*5.2.3*). They were viewed, both stained and unstained, using TEM to determine spore ultrastructure (Figures 5.10a and 5.10b). The effect of the use of different stains was also determined (Figure 5.11a, 5.11b and 5.11c).

Figure 5.10a. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in Transmit EM ™ resin, showing general ultrastructure. x 50 000 magnification. EM1648.

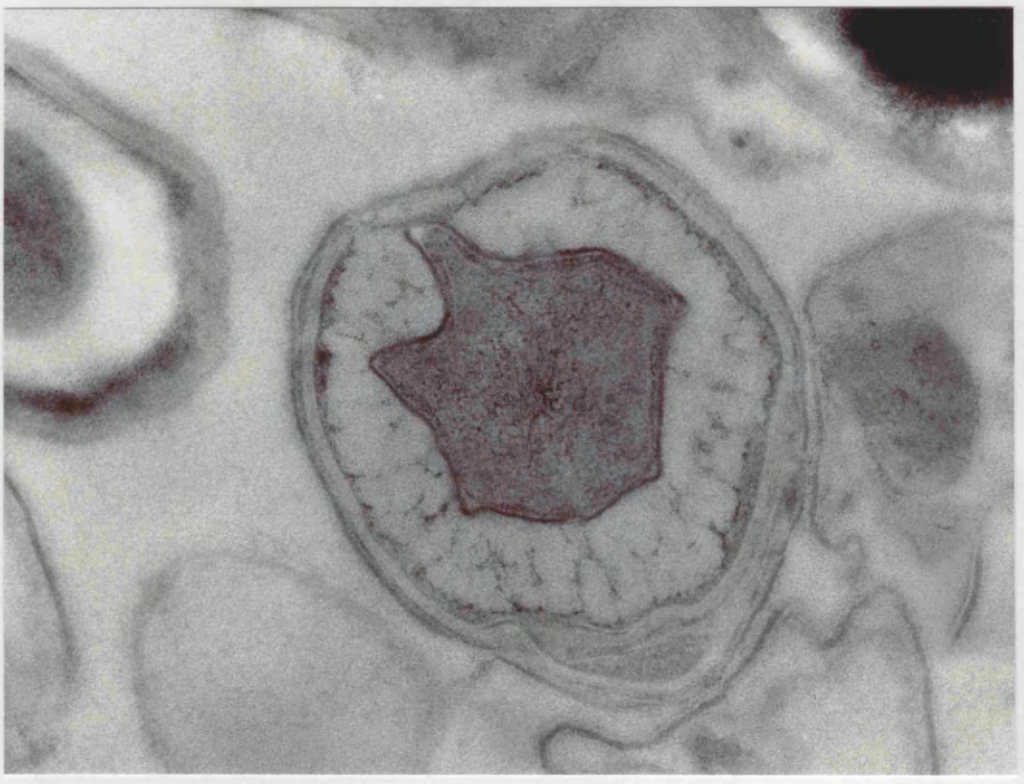


Figure 5.10b. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in Transmit EM ™ resin, showing details of spore coat layers. x 100 000 magnification. EM1660.

Figure 5.10c. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in Transmit EM ™ resin, showing poor fixation and infiltration of spore core. x 40 000 magnification. EM1476.

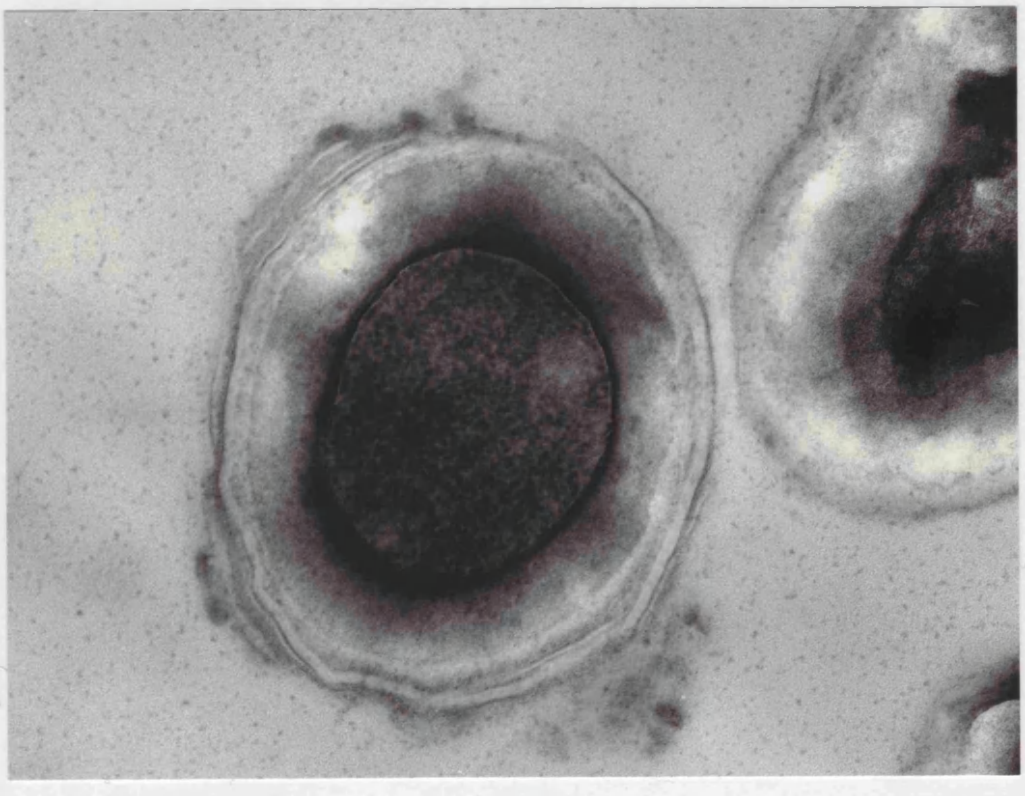


Figure 5.11a. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in Transmit EM ™ resin. Osmication, no post-staining. x 40 000 magnification. EM1787

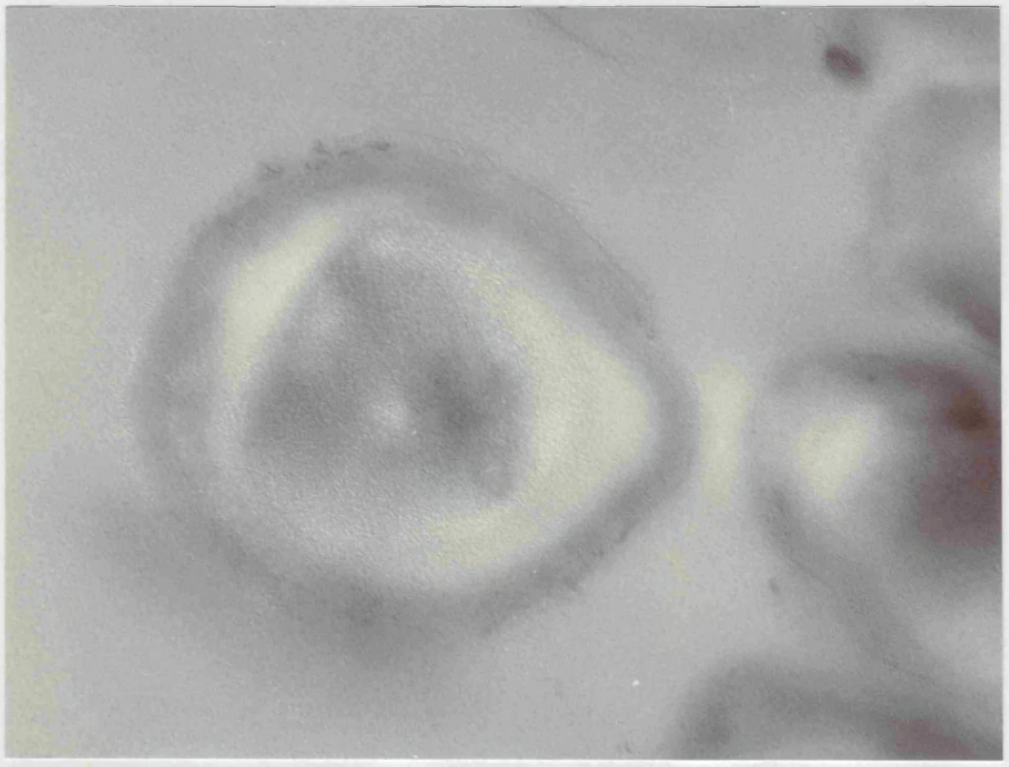


Figure 5.11b. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in Transmit EM ™ resin. Osmication and post-staining with uranyl acetate. x 40 000 magnification. EM1785.

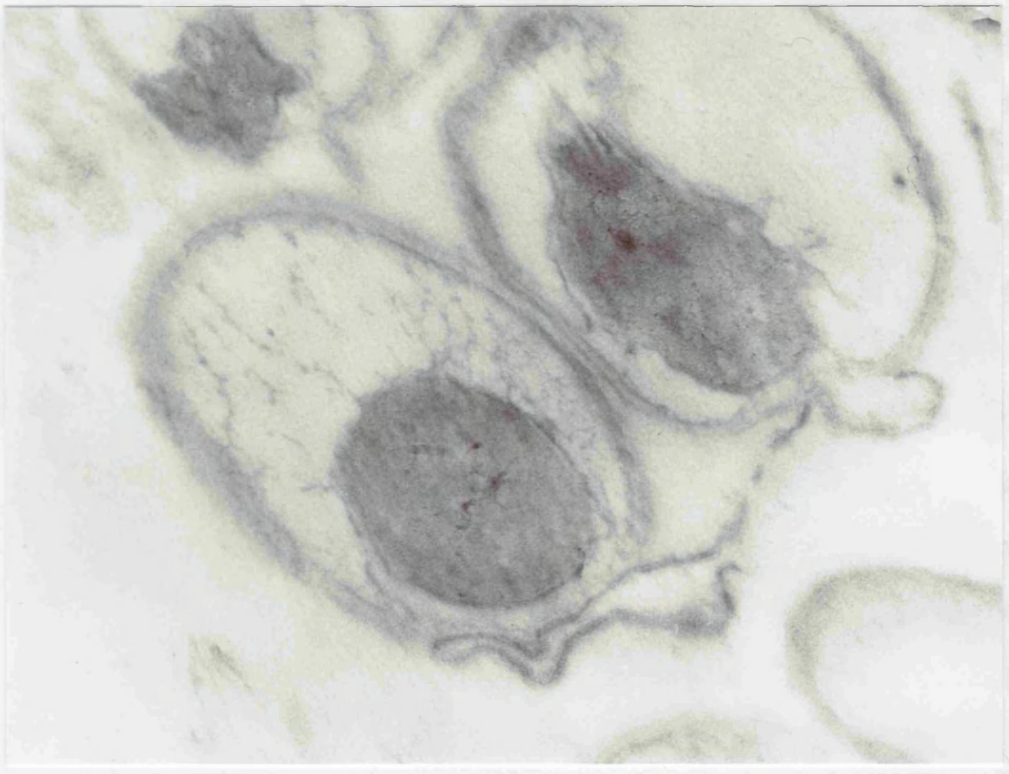


Figure 5.11c. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in Transmit EM ™ resin. Osmication and post-staining with uranyl acetate and lead citrate. x 50 000 magnification. EM1750.

5.3.4 Chemical localisation of α-glucosidase (post-embedding labelling)

Spores were prepared for TEM using LR White ™ resin (*5.2.3*). The chemical localisation reaction was carried out on thin sections (*5.2.8*). A control reaction was carried out, in which the specific substrate, naphthyl-α-D-glucoside, was omitted from the labelling solution. An untreated section from the same block was observed for comparison. Figures 5.12a, 5.12b, and 5.12c show the results of the chemical localisation reactions. There was excessive formation of precipitate in the control reaction which contained only coupling agent, but no specific substrate for the α-glucosidase enzyme. This made it difficult to interpret the results of the labelling reaction. The precipitate formed appeared to be partially soluble, affording poor localisation at the exact sites of α-glucosidase within the spores. The small dimensions of individual spores also hampered localisation, since the reaction product formed was a diffuse precipitate.

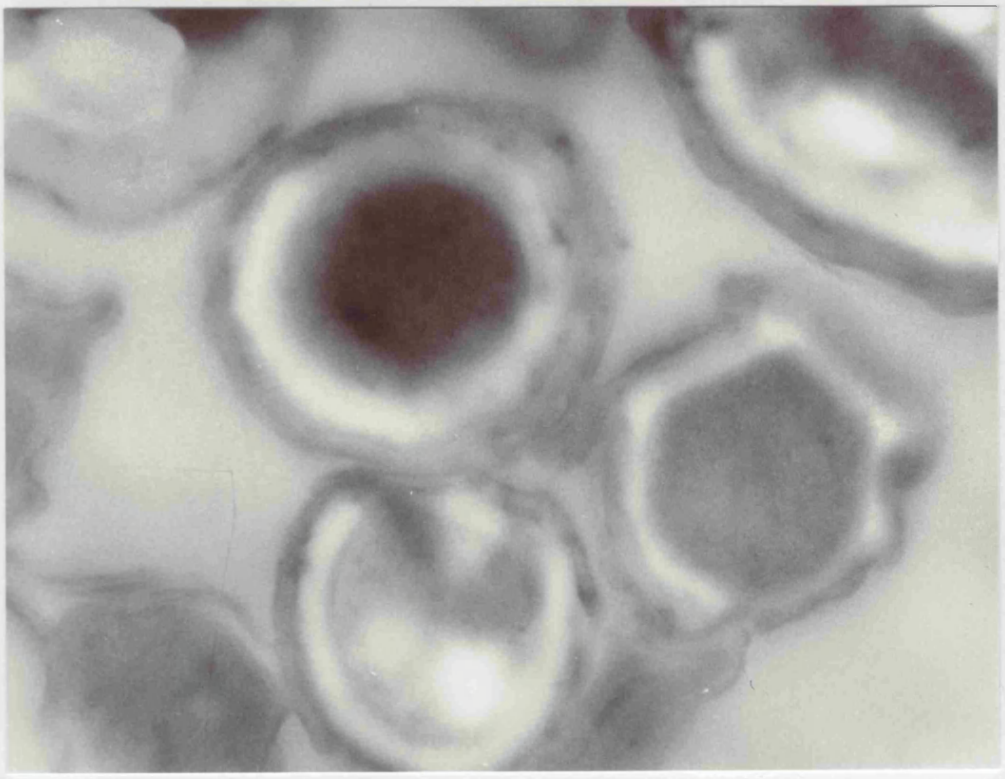


Figure 5.12a. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding chemical localisation of α-glucosidase. Control treatment - no substrate, no coupling agent. Unstained. x 60 000 magnification. EM2113.

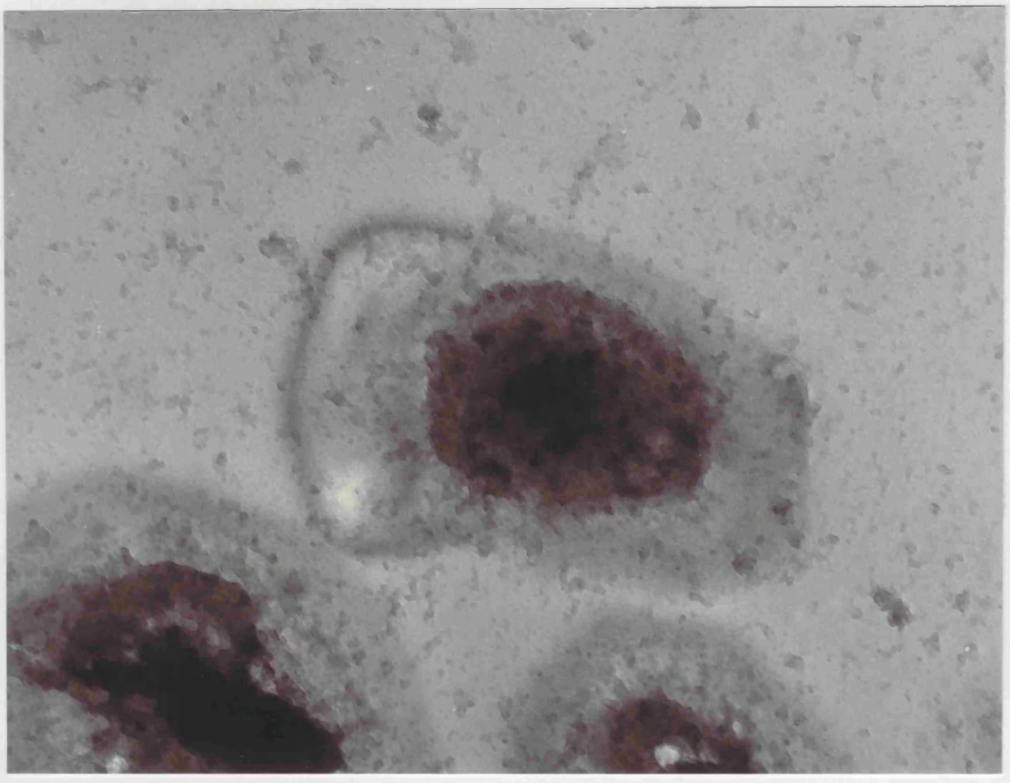
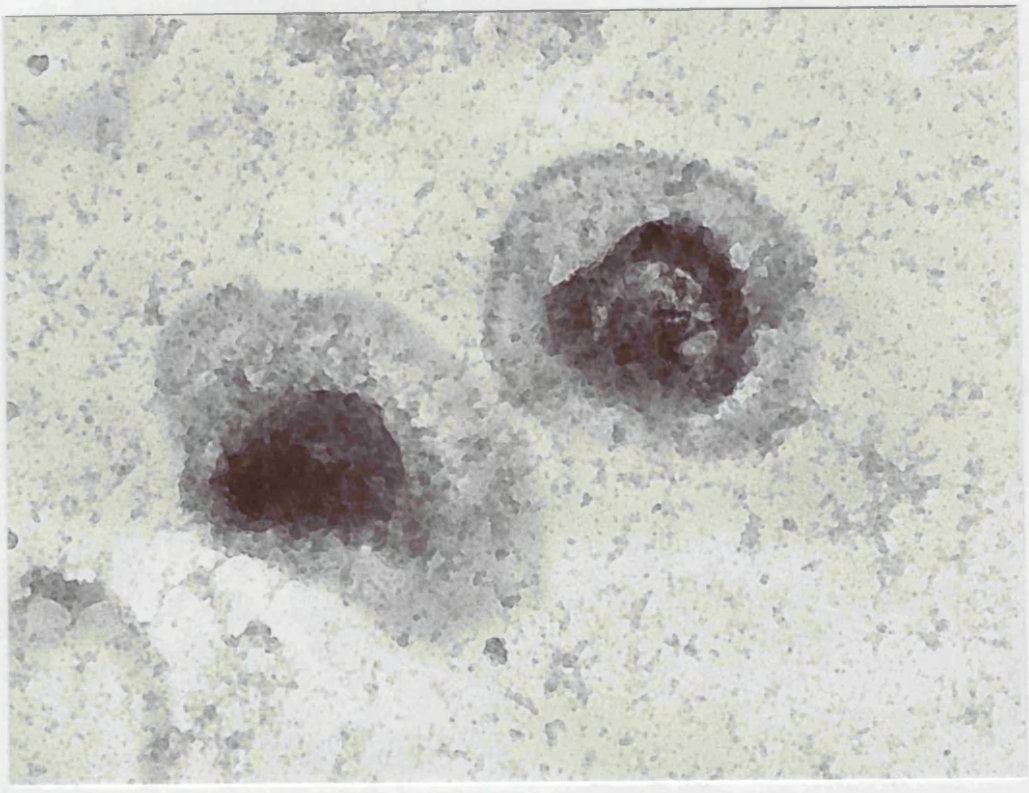


Figure 5.12b. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding chemical localisation of α-glucosidase. Control treatment, coupling agent (hexazonium pararosaniline) only. Unstained. x 40 000 magnification. EM2126.

Figure 5.12c. Transmission Electron Micrograph of thin section of *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding chemical localisation of α-glucosidase. Specific reaction with naphthyl-α-D-glucoside and coupling with hexazonium pararosaniline. Unstained. x 40 000 magnification. EM2130.



A pre-embedding labelling technique was attempted, in which the chemical localisation reaction was carried out using a spore suspension which had been fixed for 16 hours in glutaraldehyde and rinsed in phosphate buffer pH 7.4. The spores were then dehydrated and embedded in Transmit EM ™ resin in the usual manner (*5.2.2*). Results from this experiment proved inconclusive, due to the problems of localisation described above, and due to the possible solubility of the reaction product in the dehydrating and embedding media. Poor diffusion of reactants into intact spores also hindered localisation. Spores which had undergone sonication or partial

germination were used for pre-embedding labelling, to overcome problems of permeation, but with no apparent benefit.

5.3.5 Immunolocalisation of α-glucosidase

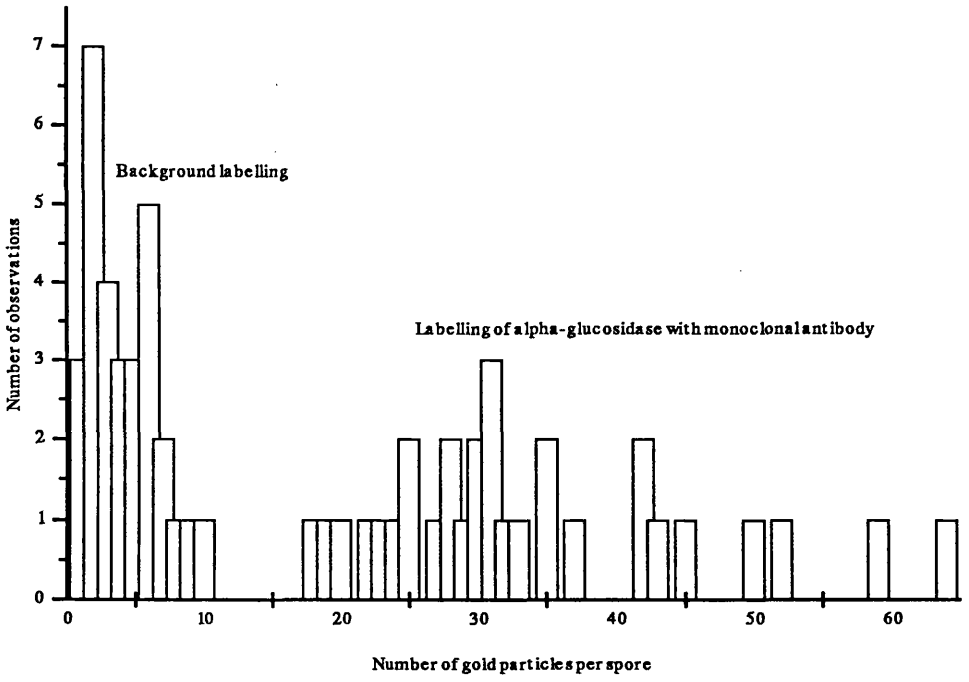
Samples of spores were fixed either by freeze drying or by glutaraldehyde (*5.2.3*). The spore samples were then prepared for TEM using the LR White procedure (*5.2.3*). The samples were polymerised in gelatin capsules using UV light (360nm) at 4°C (5.2.4.2).

Immunolocalisation reactions were carried out using the BSA / Tween 20 blocking buffer or the gelatin blocking buffer (5.2.9.2). Freshly-harvested spores and spores that had been stored for several weeks at 4°C were used.

Spores which were fixed by glutaraldehyde or by freeze drying gave very similar results. Initial reactions carried out using phosphate buffer pH 7.4 in the blocking buffer instead of Tris buffer pH 8.2, had high background labelling over the entire surface of the sections. The use of Tris buffer pH 8.2 reduced the non-specific binding of the gold-conjugate to the resin to a negligible amount. However, there remained high non-specific binding of the gold conjugate to the edges of the spores in the negative controls, which hindered interpretation of the labelled sections, since this was the postulated site of α-glucosidase activity. The use of concentrations of BSA and Tween 20 up to 1% led to a slight reduction in the non-specific binding of the gold conjugate, but also a reduction of specific binding of the monoclonal antibody. Spores that had been stored for several weeks between harvesting and fixation, and had been freeze dried, had a much reduced level of labelling on the spores, but a very high binding of the secondary antibody to the resin around the spores. Such spores that had been fixed with glutaraldehyde showed low labelling in the spores, but also a much lower level of gold binding in the resin. It is proposed that leakage of the α-

glucosidase enzyme occured during storage. The enzyme was stable following freeze drying of free enzyme in the storage buffer, which remained antigenic after embedding and polymerisation of the sample in the resin. The fixation with glutaraldehyde and subsequent rinsing and dehydration of the sample caused denaturation and removal of any free α-glucosidase associated with the other sample.

Figure 5.13. Determination of the number of gold particles bound per spore in background and spores labelled with anti-α-glucosidase monoclonal antibody



Use of the gelatin blocking buffer, containing 0.1% gelatin, led to a low level of background binding, without adversely affecting the specific labelling. A random selection of 30 spore sections was observed. The number of gold particles bound to each spore section was recorded (Figure 5.13). Figures 5.14a, 5.14b and 5.14c are

electron micrographs of the thin sections of *B.stearothermophilus* spores having undergone the immunolocalisation of α-glucosidase.

Figure 5.14a. Transmission Electron Micrograph of thin section of glutaraldehyde-fixed *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding immunolocalisation of α-glucosidase. Control reaction with 0.1% gelatin blocking buffer and secondary antibody-gold conjugate. x 40 000 magnification. EM4092.

Figure 5.14b. Transmission Electron Micrograph of thin section of freeze-dried *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding immunolocalisation of α-glucosidase. Control reaction with normal mouse serum and secondary antibody-gold conjugate using 0.1% gelatin blocking buffer. x 50 000 magnification. EM4277.

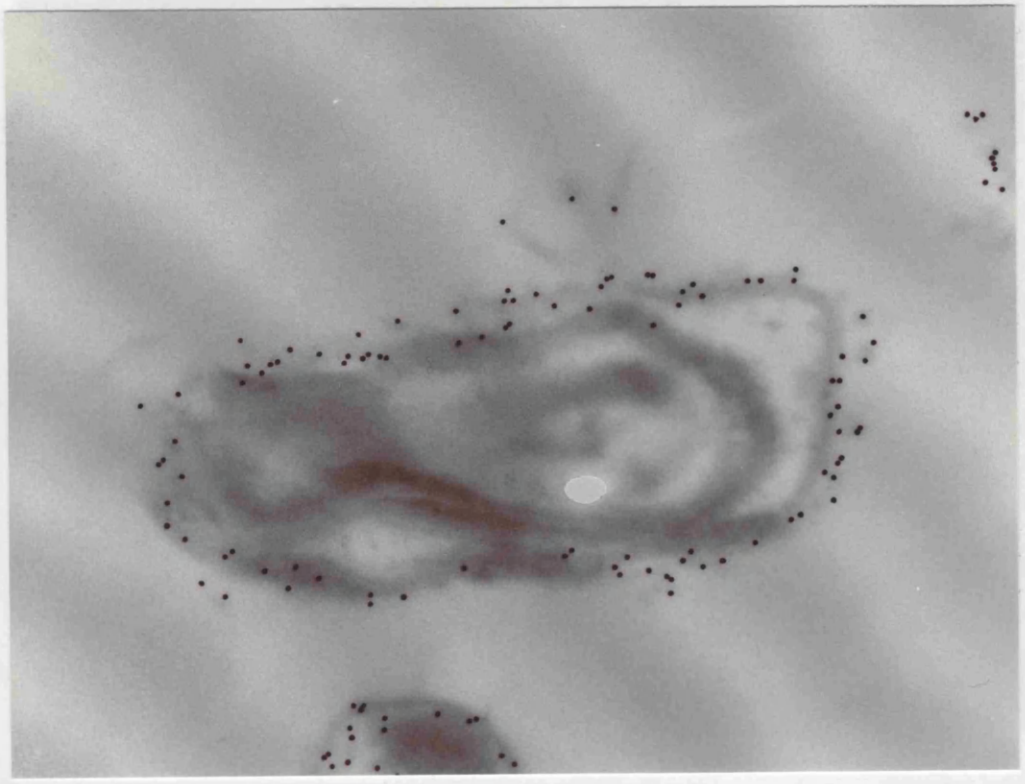


Figure 5.14c. Transmission Electron Micrograph of section of thin section of glutaraldehyde-fixed *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding immunolocalisation of α-glucosidase. Reaction with mouse anti-α-glucosidase monoclonal antibody and secondary goat anti-mouse gold-conjugated antibody (15nm).x 30 000 magnification. EM4089.

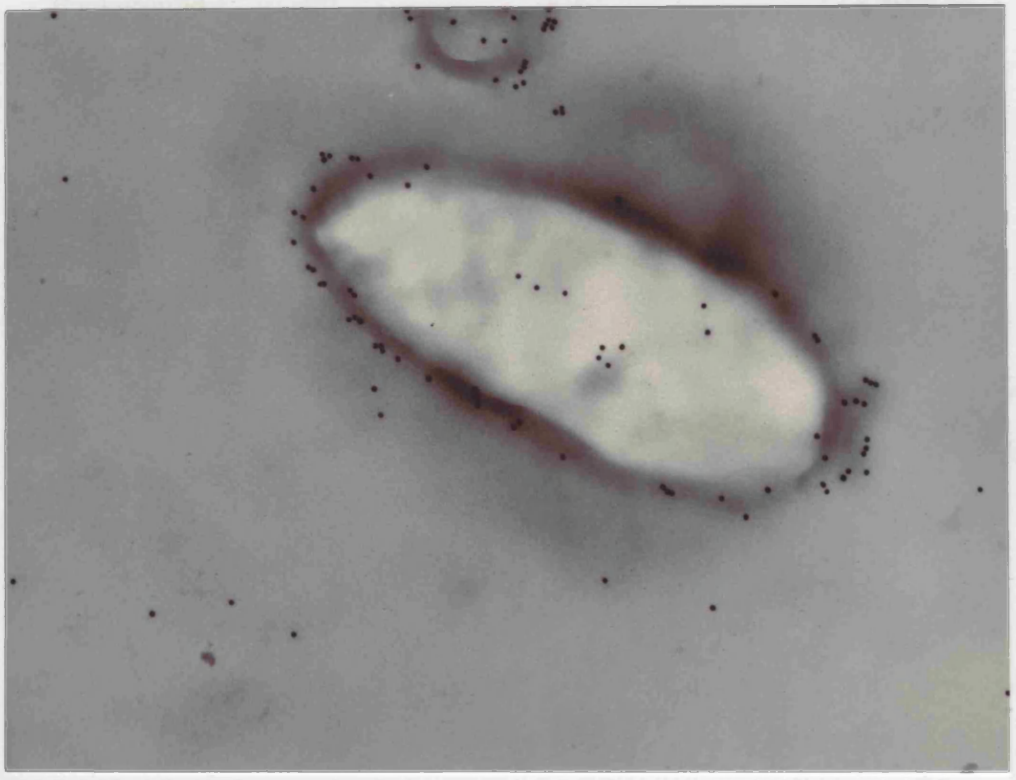


Figure 5.14d. Transmission Electron Micrograph of thin section of freeze-dried *B.stearothermophilus* spores, ATCC 7953, in LR White ™ resin. Post-embedding immunolocalisation of α-glucosidase. Reaction with mouse anti-α-glucosidase monoclonal antibody and secondary goat anti-mouse gold-conjugated antibody (15nm), using 0.1% gelatin blocking buffer. x 30 000 magnification. EM4045.

Table 5.2. Statistical data for labelling and background in immunolocalisation of α-glucosidase in *B.stearothermophilus* spores

	n	*Mean number of gold particles per spore*	*Standard deviation*
Labelled spores	30	33.7	132.98851
Background	30	4.4	5.48966

t = 13.62207

p = 1.0069×10^{-19}

A student's t-test was applied to the data, to ascertain whether the two sets of data were statistically significant (Table 5.2). At the 0.05 significance level, the two spore populations (labelled and background level) were found to be significantly different.

5.4 Discussion

Spore ultrastructure

The method for the extraction of the alpha-glucosidase enzyme involved sonication of the spore suspension. Therefore it might be expected that the enzyme had been released due to disruption of the outer spore layers. This would support the hypothesis that the enzyme resided in the spore coats only. However, electron micrographs of a spore suspension which had undergone the sonication treatment shows that individual spores are initially very different in their size, shape and surface morphology, and differ greatly in their response to sonication (Figures 5.6 - 5.8b). Reasons for this heterogeneous response may include adaptation of a spore population to a variety of germinating conditions, to ensure that the entire population of spores do not germinate synchronously. The different appearance of the untreated spores may reflect the dormant and viable sub-populations of spores, only approximately 10% of *B.stearothermophilus*, ATCC 7953, being viable under the laboratory conditions. Post-sonication, a large number of spores remain unaltered in their appearance. After 5 minutes sonication, the majority of spores are unchanged, but certain spores have a wrinkled or crushed appearance (Figure 5.7), possibly due to internal disruption of the spore by improved permeability of the spore coats. Sonication for up to 1 hour left many of the spores still unaltered visually, although a large number were significantly disrupted after this period. Figures 5.8a and 5.8b show isolated spore coats from which the inner cores have emerged, and remained as separate entities throughout the SEM preparative procedure. The heterogeneous nature of the response of the spores to sonication makes it difficult to hypothesise about the source of alpha-glucosidase within the spore, since under a given set of conditions, individual spores are at different stages of disruption.

Pre-fixation with osmium tetroxide was augmented by post-fixation with uranyl acetate and / or lead citrate. Post-fixation is preferable to pre-fixation with uranyl acetate or lead citrate since a more uniform staining is effected. Different staining protocols can be carried out on serial sections from the same block. Staining prior to embedding can also lead to hardening and problems during sectioning [172]. The spore core and outer layers of the spore appeared electron dense with osmication alone, although little membrane detail or texture of the internal structures was evident. The cortex was electron-opaque. Staining with lead citrate revealed more fine detail of the membranes. Staining with uranyl acetate revealed a network structure in the cortex of some spores and showed some detail in the core, as well as improving the detail of the spore coats. The electron density in the core is most likely to be due to binding to DNA. Uranyl acetate binds selectively to DNA rather than proteins [178, 179], the greatest selectivity being at pH 3.5. One metal ion binds for every two phosphate groups in DNA. However, certain proteins also bind strongly to uranyl ions, such as collagen. Post-staining with lead citrate and uranyl acetate following osmication gave the best results of ultrastructural detail, although very high electron density of the outer layers of the spore and the core was a problem. Clear images of the ultrastructure were important prior to attempting localisation, since chemical localisation obscures underlying ultrastructure and resins used for immunolabelling often give poorer preservation of structure than the epoxy resins.

Three to six layers of spore coats were visible in sections of spores. The network structure seen in some sections of spores has been reported to be due to changes in the pattern of staining following partial germination. The cortex usually appears featureless and electron-transparent. At the inner surface of the cortex, a more dense layer is visible which develops into the cell wall of the emergent cell when the cortex lyses [8].

The appearance of sections of spores of *B.stearothermophilus* is variable. This is partly due to the heterogeneity of a spore population, but is also caused by the alignment of spores in the resin block during sectioning. The shape and size of spore cross-sections differs greatly, but the appearance of the coat layers is reasonably constant, appearing electron dense even in unstained sections. The details of membranes seen after lead staining are almost entirely due to the presence of reduced osmium due to pre-fixation. Osmication is useful to reveal ultrastructure of spores initially. However, when chemical or immunolocalisation of enzymes is to be attempted, osmication must be omitted due to the damage of enzyme activity and antigenicity. Fixation with osmium also leads to significant loss of both lipids and proteins [180, 181, 182], which become soluble in the dehydrating and embedding media.

Problems of fixation and infiltration were evident in the processing of bacterial spores (Figure 5.9c). Longer incubation times than usual, and gradual infiltration of the resin reduced, though did not eradicate this problem. Formaldehyde is often added to the fixative solution where infiltration is a problem, since it has better penetration characteristics than glutaraldehyde. However, in this case, the use of a mixture of formaldehyde and glutaraldehyde did not improve the infiltration significantly. Poorly fixed cores resulted, and led to the centres of some spores becoming dislodged from the sections.

Chemical localisation

The sub-cellular localisation of enzymes is, by necessity, a compromise between preservation of ultrastructure and maintenance of enzyme activity [504]. Pre-embedding chemical localisation was an unsatisfactory method, due to the unpredictable uptake of the substrate, naphthyl-α-D-glucoside, and the coupling agent, hexazonium pararosaniline, into the entire volume of the spore. Therefore, post-embedding labelling of sections of spores embedded in LR White resin was the

chosen method. It was considered likely that permeation of hexazonium pararosaniline and naphthyl-α-D-glucoside into dormant bacterial spores could be problematic. However, Scherrer *et al* [78] found that the surface porosity of dormant spores of *B.cereus* strain T consisted of pores of varying sizes, but corresponded to a molecular weight of approximately 8000, and was equivalent to a hydrodynamic radius, r_{ES} = 3.2nm. The molecular weight of both reactants in the chemical localisation procedure is much lower than this value, and therefore permeation through the spore coats should be achieved. However, it has been postulated that there is a permeability barrier, the pericortex mambrane, on the inner surface of the spore coats. This may hinder permeation into the cortex and the core.

In the post-embedding chemical localisation experiments, a diffuse precipitate was formed over the entire surface of the sections, both spores and sections, in the labelled sections and the sections that had been treated with coupling agent only. It is evident that the production of the insoluble electron dense precipitate (azo-dye) is not at the site of the specific enzymatic reaction. It is difficult to be certain whether the enzyme, or the electron-dense reaction product (or intermediates) have diffused away from the original location during the preparatory procedures. This problem has been minimised, though not eliminated, by the use of modern resin systems. Errors of localisation occur if the reaction product is soluble in the dehydrating and embedding media [183]. The azo dye of a non-metallic coupling agent used in this procedure appeared diffuse and fuzzy, and completely obscures any underlying ultrastructural detail. The large size of the deposits made it difficult to put forward any probable localisation sites, especially due to the small size of the spores themselves. Another drawback of the chemical localisation method is the fixation procedure. Figure 5.9 shows that following 16 hours fixation with glutaraldehyde there remained less than 20% of the original alpha-glucosidase activity. Whilst this was sufficient for immunolocalisation, a more sensitive technique which relies on antigenicity rather than activity, this low level may preclude the use of a chemical localisation procedure.

The use of metallic coupling agents is generally more successful due to the higher electron-density and more compact nature of the deposits formed, and lower solubility in preparatory media than non-metallic azo dyes [184]. For example, electron dense deposits of non-metallic azo dyes on cell membranes could not be accurately localised to precise parts of the membrane, whereas it was possible with a metallic coupling agent [185, 186]. Chemical localisation of alpha-glucosidase using a metallic coupling agent was attempted. The synthesis of the diazonium salt of the tri (4-amino) lead phthalocyanin [187] coupling agent proved problematic, and continuation of this method was halted.

Immunolocalisation

Background labelling is the most common problem in immunocytochemistry [171, 188]. There are several contributing factors. Hydrophobic interactions of proteins in the specimen and IgG antibodies can be caused by interactions of side chains of neutral amino acids, phenylalanine, tyrosine and tryptophan. Proteins in tissue are rendered more hydrophobic by reaction with aldehyde fixatives, due to cross-linking of reactive amino acids, both within the same molecule and between adjacent protein molecules. The degree of hydrophobicity is dependent on fixation conditions, such as time, temperature and pH. Hydrophobic interaction is also a function of the type and concentration of ions. A low ionic strength buffer will help reduce the strength of hydrophobic binding. Addition of a detergent, *eg*. Tween 20, to the buffer and raising the buffer pH will reduce hydrophobic binding. The addition of a blocking protein, *eg*. bovine serum albumin, ovalbumin, gelatin, to the diluent is effective if the protein can compete with binding sites in the tissue. The sections can be incubated in the blocking protein prior to addition of the primary antibody, or it can be added to all the solutions. Ionic interactions can occur between the IgG molecules and proteins in the sample. IgG molecules have isoelectric points of between 5.8-7.3. Therefore they

will have a net negative charge at usual pH conditions. Ionic interactions will result if surface proteins on the surface of the section has a net positive charge.

Strategies used to overcome these problems are the addition of BSA blocking protein and Tween 20 detergent to the diluent. These methods reduced the non-specific background, but also reduced the specific binding of the antibody to its antigen. Although better specific labelling resulted, significant non-specific binding of gold to the outer layers of the spore was evident. This binding is thought to be due to binding of the gold conjugate to sulphur-rich proteins. The spore coats are composed of cysteine-rich proteins, similar to keratin, with which the gold-conjugated antibody will bind. In this case, the background labelling of the edges of the spores was particularly troublesome, since the spore coats are one of the possible sites of alpha-glucosidase activity. Therefore the presence of excessive background in this area hindered the interpretation of labelling experiments. This binding was minimised by the use of gelatin as a blocking agent. Gelatin is no longer a commonly-used blocking agent as it can actually worsen general background in some cases. However, when high sulphur content of proteins is a specific problem, the use of gelatin can be beneficial.

Two different fixation methods were used in preparing spores for immunocytochemical localisation; freeze drying and glutaraldehyde fixation. Glutaraldehyde fixation, the most commonly used fixation method has several advantages [171, 183, 170]. It maintained reasonable ultrastructural detail (although this is highly dependent on the resin used), but preserves sufficient enzyme activity for localisation reactions. Freeze drying avoids the problem of cross-linking of proteins to increase their stability, but preserves the proteins and other cellular constituents in a near natural state [170]. For many tissues, freeze drying would not be a suitable method since preservation of ultrastructure would be poor. However, results of immunolabelling of spores using freeze dried samples gave good results and preserved ultrastructure adequately in order to make localisation meaningful. The relatively

dehydrated state of spores was a probable explanation for the maintenance of ultrastructure, since freeze drying caused a relatively small disruption of internal structures.

Spores that had been harvested several weeks before use and stored at 4°C were initially utilised in localisation experiments. In the glutaraldehyde fixed spores, there was a very low low number of gold particles, both in the resin and attached to the spores. In the freeze dried spores, there was more labelling of the spores themselves, but a very high number of gold particles associated with the resin around the spores. It is postulated that during storage in buffer, loosely-attached alpha-glucosidase leaks into the storage medium. In the glutaraldehyde-fixed spores, the free enzyme will be washed away during rinsing and dehydration steps, and lower amount will remain attached to the spores. However, in the freeze drying process, the spore suspension containing free enzyme was frozen rapidly and freeze dried directly in the suspending buffer. Therefore, it is assumed that the enzyme was stable in a freeze dried form in the buffer, and remained antigenic during embedding and polymerisation of the resin. The labelling intensity of the freeze dried and glutaraldehyde-fixed spores was similar. It might have been expected that a higher amount of labelling would be seen in the freeze dried spores, due to cross-linking of proteins in the glutaraldehyde-fixed spores. However, although glutaraldehyde fixation reduced the activity of alpha-glucosidase substantially, the fixation apparently did not affect the antibody binding site. Indeed, studies showed that α-glucosidase activity was not affected by antibody binding, and thus the antibody binding site and active site of the α-glucosidase enzyme are presumed to be separate.

A random selection of spore sections from both labelled and control grids was observed. The number of gold particles associated with each spore was determined. There was a very large standard deviation from the means of both populations of spores. Spore populations are themselves heterogeneous. The spore sections varied

greatly in size, shape and orientation, giving vastly different available binding sites for the antibody on each section. A student's t test confirmed that there was a statistically significant difference between the two populations.

Almost all the labelling of the spores was in the outer coat layers or on the surface of the spores. This correlates well with its high concentration in extracts of spores produced by sonication, its thermostability and its postulated role in germination. The spore coats are known to have a vital function in germination. No alpha-glucosidase was associated with the cores of the spores. Pre-embedding labelling was attempted, in which freshly-harvested spores were reacted with the anti-alpha-glucosidase antibody and the secondary antibody-gold conjugate prior to dehydration and embedding of the sample. This method allowed labelling of surface antigens only, since the antibodies could not penetrate through the section. A very low labelling intensity was observed with this method, suggesting that very few antigen sites existed on the outside of the spores. The TEMs of post-embedding labelled spores show that most of the gold particles are beneath the actual surface of the spores, in the outer coat layers. The alpha-glucosidase appears to diffuse through the coat layers to the surface of the spores and into the medium on prolonged storage.

Chapter 6

GENERAL DISCUSSION AND SUGGESTIONS FOR FURTHER WORK

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One aim of this work was to optimise the α-glucosidase activity associated with the spores of *Bacillus stearothermophilus* that are used for the BI spore strips. This would have two possible benefits. Firstly, the BI would become a more sensitive monitor, and a faster readout time could be obtained. In addition, in the future, it may be desirable to develop a biochemical monitor whose function relied solely on the detection of active enzyme, that had been isolated from spores or other sources. This would remove reliance on the spores themselves, which are prone to the variability associated with any living system. For the development of such a biochemical monitor it would be necessary to optimise the production and activity of the enzyme prior to extraction and purification.

One approach to this problem is the use of strains of *Bacillus stearothermophilus* with the inherent capability of increased production of α-glucosidase compared to *Bacillus stearothermophilus* ATCC 7953 that is currently used in 1291 Attest™ Rapid Readout Biological Indicator. Suzuki *et al* [135] studied various strains of *B.stearothermophilus* and similar *Bacillus* strains, including a soil isolate, KP1006, and found that a number of the strains produced more than twice the extracellular α-glucosidase of ATCC 7953. It would be necessary to investigate the spore yields, heat resistance, growth requirements, and other characteristics of these strains prior to pursuing this option.

Large-scale production of α-glucosidase could be achieved by over-expression of the α-glucosidase gene in *B.stearothermophilus* or in other bacteria. Recent advances in thermophilic vector systems have meant that genetic manipulation of thermophilic

bacteria is now a realistic option. The ideal transformation system for *B.stearothermophilus*, and other thermophiles, would require plasmids that are stable and express antibiotic resistance at temperatures above 60-65°C. It is not known whether over-expression of the spore-derived form of the α-glucosidase enzyme, in a mesophile such as *B.subtilis*, would enable all of the characteristics of the spore enzyme to be maintained. It is possible that thermostability of the enzyme is partly determined by the location of the enzyme within the spore, and that factors such as partial dehydration and mineralisation of the spore environment may contribute to the enzyme properties. However, Fujii *et al* (6/4) expressed the structural gene for a thermostable neutral protease from *B.stearothermophilus* in both *B.subtilis* and *B.stearothermophilus*. No significant difference was found between the properties of the enzyme, including thermostability, despite the difference in host cells. Hopefully, expression of the α-glucosidase gene could be carried out in *B.subtilis*, about which knowledge and experience of genetic manipulation is widespread. This work holds great interest both from an applied aspect (ie. improved efficiency and sensitivity of the BI system), and from a purely academic standpoint, allowing the study of mechanisms of thermophily, which are still largely unknown.

It would be unlikely that even if it were possible to overexpress the enzyme in *B.stearothermophilus*, that a benefit to the product would be the result. Also, regulations covering genetically manipulated products would hinder the use of such technology. Furthermore, due to the mechanism of the readout of the biological indicator, the only real benefit would arise from addition of enzyme to the extracellular fluid prior to drying of the suspension onto the spore strips. The natural progression of this biological indicator would be towards the concept of a purely biochemical indicator, comprising purified α-glucosidase.

It would be of academic interest to sequence the α-glucosidase gene. This would allow a full comparison with similar enzymes that have been sequenced, and give

valuable information about likely active sites, mechanisms of location of the enzyme to the spore coats, and export into the extracellular medium. Mutation of the active site of α-glucosidase would allow study of the factors affecting its production and control, and the effect of the enzyme on germination.

In addition to approaches directed at the properties of spores used in the BI, the investigation of the conditions under which spores are produced and germinated also provide useful information for modification of the BI function. Further characterisation of the activity of the α-glucosidase of *B.stearothermophilus* ATCC 7953 and the nature of its control system would be valuable.

Most strains of *B.stearothermophilus* degrade starch using extracellular amylases. Maltose is generally transported into bacteria uncleaved, as a disaccharide. The best characterised system of maltose uptake is the *mal* regulon of *E.coli* [193]. This codes for a periplasmic binding protein-dependent transport system of two operons, *malEFG* and *malK-lamB-malM*. Transcription is initiated in the presence of malto-oligosaccharides. Intracellular metabolism of maltose is encoded at three other loci, including malPQ. Maltose is bound to the MalE periplasmic protein, which interacts with inner membrane proteins MalFGK, to transport the maltose across the inner membrane. A similar mechanism of uptake of maltose and other disaccharide or oligosaccharide substrates is presumed to occur in Gram positive organisms, and this is under current investigation [194].

Genes responsible for maltose utilisation from *B.stearothermophilus* ATCC 7953 were cloned in the plasmid vector pBR325 and functionally expressed in *E.coli*. The 4.2kb *B.stearothermophilus* insert suppressed the growth defects on maltose caused by mutations in *E.coli* maltose transport genes (*malE*, *malK*, or complete *malB* deletion), but did not affect mutations in genes involving intracellular maltose metabolism (*malA* region). *Bacillus stearothermophilus* ATCC 7953 is thought to

contain two uptake systems, similar to the *E.coli* uptake mechanism. The MalA protein, responsible for these effects, was poorly expressed and characterisation of its transport properties and its purification are yet to be accomplished.

A secondary aim of the immunolocalisation of the enzyme, was to follow its progress during spore during germination and outgrowth. However, although the technique was sensitive enough to allow localisation of the enzyme, the heterogeneity of any spore population makes it exceedingly difficult to observe subtle changes in individual spores. The processes of activation, germination and outgrowth do not occur synchronously in the entire spore population, and this further hinders electron microscopic study of individual spores. It is hypothesised that α-glucosidase has a role in later stages of germination and outgrowth. This is substantiated by its location in the outer spore coats and its effect in the germination of "maltose-induced" spores.

It would be beneficial to produce *B.stearothermophilus* spores in liquid culture medium, to avoid the problems of variation between batches of spores grown on complex solid media. Production of spores on solid medium is also time consuming, expensive and labour-intensive. A chemically-defined, synthetic medium for production of *B.subtilis* spores was developed, but has not been successfully modified for growth of thermophiles. The use of synthetic media is preferable due to the reduced problem of inter-batch variations, which is a common problem in complex media. The content of complex media formulations can vary significantly, affecting the characteristics of spores produced. The heat resistance of thermophilic spores produced in liquid media continues to be the main oppposition to production on liquid media.

Spores of *B.stearothermophilus* are known to be more dormant than spores of other species. Prior to heat activation, only about 10% of a population of spores will germinate and form colonies on standard media. This percentage (growth index) will

increase up to about 50% following heat activation. Beaman *et al* [100] separated activated spores, germinated spores and dormant spores into distinct fractions by bouyant density centrifugation. Activated and dormant spores will be more bouyant than dormant spores due to loss of DPA and calcium. Belliveau *et al* [195] also used this method to separate *B.megaterium* spores into 2 sub-populations. The heavier spores were significantly more heat resistant than the lighter spores, and had a higher DNA content (digenomic compared to monogenomic spores). The DPA content and Mg / Ca and Ca / DPA mole ratios did not differ significantly. Increased DNA content only occurs in a fraction of the spore population, and does not seem to be a general mechanism for spore thermoresistance.

It might be possible to separate dormant and germinated spores using this method, due to the loss of Ca and DPA from germinated spores, and thus their different densities. This could prove useful to study the differences in activity of the α-glucosidase in the two sub-populations. It is not known whether the enzyme activity is associated with germinating spores only during the incubation of the Attest units, or whether enzyme activity associated with dormant and viable spores contributes to the overall activity. Factors affecting the maintenance and breaking of dormancy in bacterial spores could be further studied.

In summary, the characterisation and localisation of the α-glucosidase enzyme in *B.stearothermophilus* ATCC 7953 spores have been accomplished. The effect of factors on the production of the enzyme in vegetative cells and spores, and its activity during spore germination have been investigated. On the strength of these findings, several approaches to the modification of the Attest ™ Rapid Readout Biological Indicator have been suggested.

References

1. Underwood, W.B. (1942). *Textbook of Sterilisation.* 2nd ed. American Steriliser Company, The Lakeside Press, R.R. Donnelly and Sons, Chicago.

2. Perkins, J.J. (1980). *Principles and Methods of Sterilisation.* 2nd ed. Charles C. Thomas, Springfield, IL. 259-260.

3. "Understanding Flash Sterilisation". Medical-Surgical Division. 3M Healthcare. St. Paul. Minnesota.

4. Pflug, I.J. and Odlaug, T.E. (1986). *Journal of Parenteral Science and Technology.* **40**, 242-248.

5. Kotilainen, H.R. and Gantz, N.M. (1987). *Infection Control.* **8**, 311-316.

6. Vesley, D., Langholz, A.C., Rohling, S.R., Foltz, W.E. (1992). *Appl. Environ. Microbiol.* **58**, 717-719.

7. Vinter, V. (1969). Physiology and Biochemistry of Sporulation. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 73-123.

8. Warth, A.D. (1978). *Adv. Microbiol. Phys.* **17**, 1-45.

9. Hanson, R.S., Curry, M.V., Gardner, J.V. and Halvorson, H.O. (1972). *Can. J. Microbiol.* **18**, 1139.

10. Warth, A.D. et al. (1963). *J. Cell Biol.* **16**, 579-592.

11. Powell, J.F. and Strange, R.E. (1953). *Biochem. J.* **54**, 205.

12. Strange, R.E. and Powell, J.F. (1954). *Biochem. J.* **58**, 80.

13. Murrell, W.G. (1969). Chemical Composition of Spores and Spore Structure. In *The Bacterial Spore.* (Gould, G.W. ed).. Academic Press Inc. (London) Ltd.

14. Rogers, H.J. (1985). Peptidoglycans : Structure, Conformation and Function in Walls and spore cortex. In *Fundamental and Applied Aspects of Bacterial Spores.* (Dring, G.J., Ellar, D.J. and Gould, G.W. ed)..Academic Press Inc.London. Ltd.

15. Warth, A.D. (1965). *Biochim. Biophys. Acta.* **101**, 315.

16. Warth, A.D., Ohye, D.F. and Murrell, W.G. (1963). *J. Cell Biol.* **16**, 579-592.

17. Aronson, A.I. and Fitz-James, P.C. (1968). *J. Mol. Biol.* **33**, 199-212.

18. Stelma, G.N., Aronson, A.I. and Fitz-James, P.C. (1980). *J. Gen. Microbiol.* **116**, 173-185.

19. Tipper, D.J. and Gauthier, J.J. (1972). Structure of the bacterial endospore. In *Spores V.* (Halvorson, H.O., Hanson, R. and Campbell, L.L. eds.). Am. Soc. Microbiol.

20. Matz, L.L., Beaman, T.C., Gerhardt, P. (1970). *J. Bacteriol.* **101**, 196-201.

21. Hodges, N.A. and Brown, M.R.W. (1975). In *Spore VI* (Gerhardt, P., Costilow, R.N. and Sadoff, H.L. eds.). pp550-554. American Society of Microbiology, Washington.

22. Cheung, H.Y., Vitkovic, L. and Brown, M.R.W. (1982). *J. Gen. Microbiol.* **128**, 2403-2409.

23. Warth, A.D. (1985). Mechanisms of heat resistance. In *Fundamental and Applied Aspects of Bacterial Spores.* (Dring, G.J., Ellar, D.J. and Gould, G.W. ed)..Academic Press Inc.London. Ltd.

24. Srivastava, O.P. and Fitz-James, P.C. (1981). *Can. J. Microbiol.* **27**, 408-416.

25. Russell, A.D. (1982). The Destruction of Bacterial Spores. Academic Press Inc. (London) Ltd.

26. Bernlohr, R.W. and Leitzman, C. (1969). Control of sporulation. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 183-213.

27. Lechowich, R.V. and Ordal, Z.J. (1962). *Can. J. Microbiol.* **8**, 287.

28. Falcone, G., Armani, G., Previtera, A. and Bertini, V. (1966). *Boll. Ist. Sieroter. Milanese.* **45**, 11.

29. Halvorson, H.O. (1957). *J. Appl. Bact.* **20**, 305.

30. Grossman, A.D. and Losick, R. (1988). *Proc. Natl. Acad. Sci. USA.* **85**, 4369-4373.

31. Young, I.E. and Fitz-James, P.C. (1959). *J. Biophys. Biochem. Cytol.* **6**, 467-481.

32. Young, I.E. and Fitz-James, P.C. (1959). *J. Biophys. Biochem. Cytol.* **6**, 483-498.

33. Fitz-James, P.C. and Young, E. (1969). Morphology of Sporulation. In *The Bacterial Spore*. Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 39-72.

34. Murrell, W.G., Ohye, D.F. and Gordon, R.A. (1969). Cytological and chemical structure of the spore. In *Spores IV*. Campbell, L.L. (ed.). Am. Soc. Microbiol. 1-19.

35. Aronson, A.I. and Fitz-James, P.C. (1968). *J. Mol. Biol.* **33**, 199-212.

36. Jenkinson, H.F. and Mandelstam, J. (1985). In *Fundamental and Applied Aspects of Bacterial Spores*. Dring, G.J., Ellar, D.J. and Gould, G.W. (ed.).Academic Press Inc.(London) Ltd.

37. Hanson, R.S., Peterson, J.A. and Yousten, A.A. (1970). *Ann. Rev. Microbiol.* **24**, 53-90.

38. Murrell, W.G. (1967). *Advan. Microbial Phys.* **1**, 133-251.

39. Errington, J. (1993). *Microbiol. Rev.* **57**, 1-33.

40. Zheng, L. Halberg, R., Roels, S., Ichikawa, H., Kroos, L. and Losick, R. (1992). *J. Mol. Biol.* **226**, 1037-1050.

41. Jenkinson, H.F. (1981). *J. Gen. Microbiol.* **127**, 81-91.

42. Jenkinson, H.F. (1983). *J. Gen. Microbiol.* **129**, 1945-1958.

43. Burbulys, D., Trach, K.A., Hoch, J.A. (1991). *Cell.* **64**, 545-552.

44. Hoch, J.A. (1970). *Adv. Genet.* **18**, 69-99.

45. Ronson, C.W., Nixon, B.T. and Austubel, F.M. (1987). *Cell.* **49**, 579-581.

46. Perego, M., Cole, S.P., Burbulys, D., Trach,K. and Hoch, J.A. (1989). *J. Bacteriol.* **171**, 6187-6196.

47. Trach, K. and Hoch, J.A. (1989). *J. Bacteriol.* **171**, 1362-1371.

48. Losick, R. and Stragier, P. (1992). *Nature (London).* **355**, 601-604.

49. Margolis, P., Driks, A. and Losick, R. (1991). *Science.* **254**, 562-565.

50. Setlow, P. (1988). *Annu. Rev. Microbiol.* **42**, 319-338.

51. Errington, J. and Mandelstam, J. (1984). *J. Gen. Microbiol.* **130**, 2115-2121.

52. Jenkinson, H.F., Sawyer, W.D. and Mandelstam, J. (1981). *J. Gen. Microbiol.* **123**, 1-16.

53. Zheng, L., Donovan, W.P., Fitz-James, P.C. and Losick, R. (1988). *Genes Dev.* **2**, 1047-1054.

54. Keynan, A. and Evenchik, Z. (1969). Activation. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 358-396.

55. Hills, G.M. (1949). *Biochem. J.* **45**, 353.

56. Powell, J.F. and Hunter, J.R. (1955). *J. Gen. Microbiol.* **13**, 59.

57. Hyatt, M.T. and Levinson, H.S. (1962). *J.Bacteriol.* **82**, 706.

58. O'Connor, R.J. and Halvorson, H.O. (1961). *J. Bacteriol.* **82**, 706.

59. Crook, P.G. (1952). *J. Bacteriol.* **63**, 193.

60. Murrell, W.G. (1955). " The bacterial endospore" Monograph, Univ. Sydney, Australia.

61. Halvorson, H.O. and Church, B.D. (1958). In *Spores* (H.O. Halvorson ed.). Burgess Publ. Co. Minneapolis, MN, USA. 359.

62. Krask, B.J. (1956). Thesis, Univ. Chicago.

63. Curran, H.R. and Evans, F.R. (1945). *J. Bacteriol.* **49**, 335.

64. Finley, N and Fields, M.L. (1962). *Appl. Microbiol.* **10**, 23.

65. Gould, H.W. and Hitchins, A.D. (1963). *J. Gen. Microbiol.* **33**, 413.

66. Kenyan, A., Evenchik, Z., Halvorson, H.O. and Hastings, J.W. (1964). *J. Bacteriol.* **88**, 313.

67. Hitchins, A.D., Gould, G.W. and King, W.L. (1966). *J. Appl. Bact.* **29**, 505.

68. Gould, G.W. (1969). Germination. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 397-444.

69. Hills, G.M. (1949). *Biochem. J.* **45**, 353.

70. Hills, G.M. (1949). *Biochem. J.* **45**, 363.

71. Hills, G.M. (1950). *J. Gen. Microbiol.* **4**, 38.

72. Levinson, H.S. and Hyatt, M.T. (1966). *J. Bacteriol.* **91**, 1811-1818.

73. Dring, G.J. and Gould, G.W. (1971). *J. Gen. Microbiol).* **65**, 101-104.

74. Lewis, J.C., Snell, N.S. and Burr, H.K. (1960). *Science.* New York. **132**, 544-545.

75. Gould, G.W. and King, W.L. (1969). In *Spores* Vol. 4. Edited by L.L. Campbell, Washington D.C. *Am. Soc. Microbiol.* 276-286.

76. Yasuda, Y. and Tochikubo, K. (1984). *Microbiol. Immunol.* **28**, 197-207.

77. Watabe, K., Nishihara, T. and Kondo, M. (1974). *Japan. J. Microbiol.* **18**, 173-180.

78. Scherrer, R., Beaman, T.C. and Gerhardt, P. (1971). *J. Bacteriol.* **108**, 868-873.

79. Stewart, G.S.A.B., Johnstone, K., Hagelberg, E. and Ellar, D.J. (1981). *Biochem. J.* **198**, 101-106.

80. Yasuda, Y. and Tochikubo, K. (1985). *Microbiol. Immunol.* **29**, 1011-1017.

81. Tochikubo, K. and Yasuda, Y. (1985). *Microbiol. Immunol.* **29**, 213-228.

82. Tochikubo, K. (1971). *J. Bacteriol.* **108**, 652-661.

83. Sano, K., Otani, M., Vehara, R., Kumura, M. and Umezawa, C. (1988). *Microbiol. Immunol.* **32**, 877-885.

84. Setlow, P. and Kornberg, A. (1970). *J. Biol. Chem.* **245**, 3637-3644.

85. Strauss, N. (1983). *FEMS Microbiol. Lett.* **20**, 379-384.

86. Sano, K., Otani, M., Umezawa, C. and Nakatani, Y. (1994). *Biosci. Biotech. Biochem.* **58**, 931-933.

87. Irie, R., Okamoto, T. and Fujita, Y. (1982). *J. Gen. Appl. Microbiol.* **28**, 345-354.

88. Shay, L.K. and Vary, J.C. (1978). *Biochim. Biophys. Acta.* **538**, 284-292.

89. Moir, A., Yazdi, M.A. and Kemp, E.H. (1991). *Res. Microbiol.* **142**, 847-850.

90. Moir, A. and Smith, D.A. (1990). *Annu. Rev. Microbiol.* **44**, 531-553.

91. Sammons, R.L., Moir, A. and Smith, D.A. (1981). *J. Gen. Microbiol.* **124**, 229-241.

92. Taylor, R.F. (1984). *Microbiol. Rev.* **48**, 181-198.

93. Hyatt, M.T. and Levinson, H.S. (1957). *J. Bacteriol.* **74**, 87.

94. Gould, G.W. (1962). *J. Appl. Bact.* **25**, 35.

95. Strange, R.E. and Hunter, J.R. (1969). Outgrowth and the Synthesis of Macromolecules. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd.

96. Vinter, V. and Slepecky, R.A. (1965). *J.Bacteriol.* **90**, 803.

97. Woese, C.R. (1959). *J. Bacteriol.* **77**, 690.

98. Bender, G.R. and Marquis, R.E. (1985). *Appl. Environ. Microbiol.* **50**, 1414-1421.

99. Beaman, T.C. and Gerhardt, P. (1986). *Appl. Environ. Microbiol.* **52**, 1242-1246.

100. Beaman, T.C., Greenamyre, J.T., Corner, T.R., Pankratz, H.S. and Gerhardt, P. (1982). *J. Bacteriol.* **150**, 870-877.

101. Beaman, T.C., Pankratz, H.S. and Gerhardt, P. (1988). *Appl. Environ. Microbiol.* **54**, 2515-2520.

102. Lewis, J.C., Snell, N.S. and Burr, H.K. (1960). *Science.* **132**, 544.

103. Freisen, W.J. and Anderson, R.A. (1974). *Can. J. Pharm. Sci.* **9**, 50-53.

104. Cook, A.M. and Gilbert, R.J. (1968). *J. Food Technol.* **3**, 295-302.

105. Aoki, H. and Slepecky, R.A. (1973). *J. Bacteriol.* **114**, 137-143.

106. Tallentire, A. and Chiori, C.D. (1963). *J. Pharm. Pharmacol.* **15**, 148T-149T.

107. Molin, G. and Svensson, M. (1976). *Antonie van Leeuwenhoek.* **42**, 387-395.

108. El-Bisi, H.M. and Ordal, Z.J. (1965). *J. Bacteriol.* **71**, 1-9.

109. Levinson, H.S. and Hyatt, M.T. (1964). *J. Bacteriol.* **87**, 876-886.

110. Beloian, A. (1977). In *Disinfection, Sterilisation and Preservation.* 2nd Ed. Block, S.S. ed. Lea and Febiger, Philadelphia, 11-49.

111. Gorman, S.P., Scott, E.M. and Hutchinson, E.P. (1984). *J. Appl. Bact.* **57**, 153-163.

112. Kornberg, A., Spudich, J.A., Nelson, D.L. and Deutscher, M.P. (1968). *Ann. Rev. Biochem.* **37**, 51-78.

113. Sadoff, H.L. (1969). Spore enzymes. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 275-299.

114. Doi, R. (1969). Changes in nucleic acids during sporulation. In *The Bacterial Spore.* Gould, G.W. (ed.). Academic Press Inc. (London) Ltd. 125-166.

115. Murrell, W.G. (1952). PhD thesis. University of Oxford.

116. Sadoff, H.L. (1970). *J. Appl. Bact.* **33**, 130-140.

117. Amaki, Y., Nakano, H. and Yamane, T. (1994). *Appl. Microbiol. Biotechnol.* **40**, 664-668.

118. McRee, D.E., Redford, S.M., Getzoff, E.D., Lepock, J.R., Hallewell, R.A., Tainer, J.A. (1990). *J. Biol. Chem.* **265**, 14234-14241.

119. Volkin, D.B. and Klibanor, A.M. (1987). *J. Biol. Chem.* **262**, 2945-2950.

120. Suzuki, Y. (1989). *Proc. Jpn. Acad.* **65**, Ser B, 146-148.

121. Suzuki, Y., Oishi, K., Nakano, H. and Nagayama, T. (1987). *Appl. Microbiol. Biotechnol.* **26**, 546-551.

122. Watanabe, K., Kitamura, K., Iha, H. and Suzuki, Y. (1990). *Eur. J. Biochem.* **192**, 609-620.

123. Watanabe, K., Chishiro, K., Kitamura, K. and Suzuki, Y. (1991). *J. Biol. Chem.* **266**, 24287-24294.

124. Kelly, C.T. and Fogarty, W.M. (1983). *Process Biochem.* **18**, 6-12.

125. Kelly, C.T., Giblin, M. and Fogarty, W.M. (1986). *Can. J. Microbiol.* **32**, 342-347.

126. Urlaub, H and Wöber, G. (1978). *Biochim. Biophys. Acta.* **522**, 161-173.

127. Sata, H, Taniguchi, H and Maruyama, Y. (1987). *Agric. Biol. Chem.* **51**, 3275-3280.

128. Suzuki, Y., Ueda, Y., Nakamura, N and Abe, S. (1979). *Biochim. Biophys. Acta.* **566**, 62-66.

129. Nakao, M., Nakayama, T., Harada, M., Kakudo, A., Ikemoto, H., Kobayashi, S. and Shibano, Y. (1994).*Appl. Microbiol. Biotechnol.*

130. Nakao, M., Nakayama, T., Kakudo, A., Inohara, M., Harada, M., Omura, F. and Shibano, Y. (1994). *Eur. J. Biochem.* **220**, 293-300.

131. Suzuki, Y and Shinji, M. (1987). *Starch.* **39**, 271-273.

132. Suzuki, Y., Aoki, R and Hayashi, H. (1982). *Biochim. Biophys. Acta.* **704**, 476-483.

133. Suzuki, Y., Kishigami, T and Abe, S. (1976). *Appl. Environ. Microbiol.* **41**, 807-812.

134. Suzuki, Y., Shinji, M and Eto, N. (1984). *Biochim. Biophys. Acta.* **787**, 281-289.

135. Suzuki, Y, Yuki, J., Kishigami, T and Abe, S. (1976). *Biochim. Biophys. Acta.* **445**, 386-397.

136. Gottschalk, A. In *The Enzymes: Chemistry and Mechanisms of Action.* (1950). Summer, J.B. and Myrback, K. (eds.). 1st Edition. Volume 1. Academic Press Inc. (New York) Ltd. 551.

137. MacGregor, E.A. and Svensson, B. (1989). *J. Biochem.* **259**, 145-152.

138. Svensson, B. (1988). *FEBS Lett.* **230**, 72-76.

139. MacGregor, E.A. (1993). *Starch.* **45**, 232-237.

140. MacGregor, E.A. (1988). *J. Protein Chem.* **7**, 399-415.

141. Steele, G.C. (1987). Draft protocol for the production of *B.stearothermophilus* spores for use as biological indicators for LTSF sterilisation. LTSF Reference Laboratory. Luton College.

142. Documenta Geigy Scientific Tables. (1962). 6th Edition. Geigy Pharmaceutical Company Ltd. Manchester.

143. Long, S.K. and Williams, O.B. (1958). *J. Bacteriol.* **76**, 332.

144. Lowry, O. (1951). *J. Biol. Chem.* **193**, 265.

145. Eisenthal, R and Danson, M.J.(eds.). Enzyme Assays. A Practical Approach. (1992). Oxford University Press.

146. Cornish-Bowden, A. and Eisenthal, R. (1974). *Biochem. J.* **139**, 721.

147. Eisenthal, R. and Cornish-Bowden, A. (1974). *Biochem. J.* **139**, 715-720.

148. Sambrook, J., Fritsch, E. and Maniatis, T. (1989). Molecular Cloning. A Laboratory Manual. 2nd Ed. Cold Spring Harbour Laboratory Press, New York.

149. Welker, N.E. and Campbell, L.L. (1963). *J. Bacteriol.* **86**, 681-686.

150. Mayer, F.C. and Larner, J. (1959). *J. Am. Chem. Soc.* **81**, 188-193.

151. Randall, L.L. and Hardy, S.J.S. (1984). *Microbiol. Rev.* **57**, 290-298.

152. Blobel, G. and Dobberstein, B. (1975). *J. Cell Biol.* **67**, 835-851.

153. Blobel, G. and Sabatini, D.D. (1971). In *Biomembranes* (L.A. Manson ed). vol. 2. Plenum Publishing Corp. New York. 193-196.

154. Francisco, J.A., Earhart, C.F. and Georgiou, G. (1992). *Proc. Natl. Acad. Sci. USA.* **89**, 2713-2717.

155. Schatz, P.J. and Beckwith, J. (1990). *Annu. Rev. Genet.* **24**, 215-248.

156. Wickner, W. (1979). *Annu. Rev. Biochem.* **48**, 23-45.

157. Beckwith, J. and Ferro-Novick, S. (1986). *Curr. Top. Microbiol. Immunol.* **125**, 5-27.

158. Hoffman, C. and Wright, A. (1985). *Proc. Natl. Acad. Sci. USA.* **82**, 5107-5111.

159. Tommassen, J., van Tot, H. and Lutenberg, B. (1983). *EMBO J.* **2**, 1275-1279.

160. Boeke, J.D. and Model, P. (1982). *Proc. Natl. Acad. Sci. USA.* **79**, 5200-5204.

161. Pardo, J.M., Ianez, E., Zalacain, M., Claros, M.G. and Jimenez, A. (1988). *FEBS Lett.* **239**, 179-184.

162. Kobayashi, Y., Steinberg, W., Higa, A., Halvorson, H.O., and Levinthal, C. (1965). In *Spores III*. Halvorson, H.O. (ed.). Burgess Publishing Co., Minneapolis. 200-212.

163. Thompson,P.J. and Thames,O.A. (1967. *Appl. Microbiol.* **15**, 975-979.

164. Donellan, J.E, Nags, E.H and Levinson, H.S. (1964). *J. Bacteriol.* **87**; 332-336.

165. Srivastava, O.P. and Fitz-James, P.C. (1981). *Can. J. Microbiol.* **27**, 408-416.

166. Welker, N.E. and Campbell, L.L. (1963). *J. Bacteriol.* **86**, 687-691.

167. Terui, G., Okada, H. and Oshuma, Y. (1959). Studies on the correlation of alpha-glucosidase formation with genotype composition in *Saccharomyces*. (I). *Technol. Rep. Osaka Univ.* **9**, 237-259.

168. Newman, G.R. (1989). LR White Embedding Medium for Colloidal Gold Methods *Colloidal Gold : Principles, Methods and Applications*. Vol. 2. Hayat, M. ed... San Diego Academic Press Inc. 47-73.

169. Hall, J.L. and Hawes, C. (1991). Electron Microscopy of Plant Cells. Academic Press Inc., London.

170. Hayat, M.A. Principles and Techniques of Electron Microscopy. Biological Applications. 3rd Ed. Macmillan Press Ltd., London.

171. Hunter, E. Practical Electron Microscopy. A Beginner's Illustrated Guide. 2nd Ed. (1993). Cambridge University Press, Cambridge.

172. Trump, B.F., Smuckler, E.A. and Benditt, E.P. (1961). *J. Ultrastruct. Res.* **5**, 343.

173. Peachey, L.D. (1958). *J. Biophys. Biochem. Cytol.* **5**, 511.

174. Lehrer, G.M. and Ornstein, J.E. (1959). *J. Biophys. Biochem. Cytol.* **6**, 399.

175. Hayashi, M., Shirahama, T. and Cohen, A.S. (1968). *J. Cell Biol.* **36**, 287.

176. Barka, T. (1960). *Nature.* **187**, 248.

177. Davis, B.J. and Ornstein, L.J. (1959). *J. Histochem. Cytochem.* **7**, 297.

178. Zobel, C.R. and Beer, M. (1961). *J. Biophys. Biochem. Cytol.* **10**, 335.

179. Seligman, A.M., Wasserkrug, H.L. and Hanker, J.S. (1966). *J. Cell Biol.* **30**, 424.

180. Bahr, G.F. (1955). *Electron stains IV. Exptl. Cell Res.* **9**, 277.

181. Dallam, R.D. (1957). *J. Histochem. Cytochem.* **5**, 178.

182. Wood, R.L. and Luft, J.H. (1963). *J. Cell Biol.* 19, 83A.

183. Scarpelli, D.G. and Kanczak, N.M. (1965). *Intern. Rev. Exptl. Path.* **4**, 55-126.

184. Bowen, I.D. (1973). Glycosidases, β-glucosidases, β-glucuronidase. In *Electron Microscopy of Enzymes: Principles and Methods*. Hayat, M.A. (ed.). Van Nostrand Reinhold Ltd. New York. 77-103.

185. Barnett, R.J. (1959). *Exptl. Cell Res. Suppl.* **7**, 65.

186. Pepe, F.A., Finck, H. and Holtzer, H. (1961). *J. Biophys. Biochem. Cytol.* **11**, 533.

187. Livingston, D.C., Coombs, M.M., Franks, L.M., Maggi, V. and Gahan, P.B. (1969). *Histochemie.* **18**, 48-60.

188. Naish, S.J. (ed.). (1989). Handbook of Immunochemical Staining Methods. DAKO Corporation, Carpinteria, California.

189. Wu, L. and Welker, N.E. (1989). *J. Gen. Microbiol.* **135**, 1315-1324.

190. Hoshino, T., Ikeda, T., Narushima, H. and Tomizuka, N. (1985b). *Can. J. Microbiol.* **31**, 339-345.

191. Hoshino, T., Ikeda, T., and Tomizuka, N. (1985d). Temperature dependency of plasmid maintenance and gene expression in *Bacillus subtilis* and *Bacillus stearothermophilus*. *Report of the Fermentation Research , Japan.* **64**, 29-41.

192. Fujii, M, Takagi, M, Imanaka, T and Aiba, S. (1983). *J. Bacteriol* **154**, 831-837.

193. Schwartz, M. (1987). The maltose regulon. In Neidhart, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., Umbarger, H.E. *Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology. *Am. Soc. Microbiol.* 1482-1502.

194. Liong, E.C. and Ferenci, T. (1994). *Mol. Gen. Genet.* **243**, 343-352.

195. Belliveau, B.H., Beaman, T.C. and Gerhardt, P. (1990). *Appl. Environ. Microbiol.* **56**, 2919-2921.

Appendix 1

Amino-terminal sequence of spore alpha-glucosidase (Alta Bioscience, The University of Birmingham)

Residue			
1	ala (A0	met (M)	gly (G)
2	ala (A)		
3	pro (P)	asp (D)	ala (A) ?
4	tyr (Y)	phe (F)	
5	gly (G)	tyr (Y)	
6	gly (G)		
7	val (V)	gly (G)	
8	phe (F)	val (V)	
9	ala (A)		
10	leu (L)		

There were reported to be at least two sequences present. They are arranged in descending order of abundance at each residue. The general background was high.